The Role of the Hedgehog Signaling Pathway in the Regulation of Larval and Adult Appendage Patterning in the Flour Beetle, Tribolium Castaneum

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The Role of the Hedgehog Signaling Pathway in the Regulation of Larval and Adult Appendage Patterning in the Flour Beetle, *Tribolium castaneum*

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Submitted in Partial Fulfillment of the Prerequisite for Honors in Biological Sciences

April 2014
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ACKNOWLEDGEMENTS

The completion of this project would not have been possible without the support and effort of many people. First and foremost, to Professor Yuichiro Suzuki, my thesis advisor, mentor, and friend, words are not enough to express how grateful I am to you. You had faith in me when I had none and gave me direction when I was lost. Your dedication, excitement and passion for science are truly inspiring and contagious. All I have achieved, in life and in school, especially the development of this thesis, is due to your teachings and constant encouragement. It has been an honor and pleasure working under your guidance.

To the Suzuki lab, thank you for all the encouragement and laughter. In particular, to Nicole Hatem, Jacquelyn Chou, and Vicky Wang, thank you for your unwavering support and help with this project. I would also like to express my gratitude to Carla Villareal, whose own thesis work inspired this particular project. To Amy Ko, my fellow thesising student, there’s no one else I’d rather go through this process with than you. Thank you for your friendship and for being by my side.

I cannot express enough thanks to my thesis committee members, Professor Kimberly O’Donnell, Professor Michelle LaBonte and my honors visitor, Professor Robert Berg for their guidance throughout this process. Thank you for being a constant source of inspiration and for the preliminary reading of this manuscript. In addition, a thank you to Professor Emily Buchholtz, your encouragement and passion for evolutionary biology is greatly appreciated.

I would like to give special thanks to Professor David Ellerby. Despite my lack of experience in research sophomore year, you took me under your wing. You have my sincerest gratitude for sparking my passion for research and for giving me the skills and confidence I needed during my college life.

Finally, to my family, friends, and taiko group, Aiko, thank you for your continued support and love throughout my endeavors. Thank you for being my anchor, sail, and compass.

I dedicate this thesis to the aforementioned people. My accomplishments, in academics and in life, all belong to you.
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ABSTRACT

A key innovation in insect evolution is complete metamorphosis, which is characterized by the development of specialized larval appendages. To gain insights into the origins of these larval appendages, the role of the Hedgehog (Hh) signaling during larval development and metamorphosis was examined. Using the red flour beetle, Tribolium castaneum, Hh signaling was silenced to determine its effects on cell proliferation. Downregulation of Hh signaling resulted in disrupted cell proliferation during metamorphosis. In addition, downregulation of Patched and Costal-2, which act as antagonists of the Hh signaling pathway, led to larval limb tissue overgrowth. Our findings indicate that Hh plays a major role in regulating growth and morphogenesis of larval appendages and suggest that co-option of Hh signaling during insect evolution may have led to the evolution of imaginal cells.
INTRODUCTION

**Evolution of insect metamorphosis**

An incredible amount of morphological diversity and number of species exist on this planet. How such morphological diversity evolves is one of the major questions in evolutionary developmental biology. A particularly puzzling phenomenon that remains poorly understood is the evolution and mechanisms underlying metamorphosis. Insects, which make up the majority of all animal species, made the shift to complete metamorphosis around 300 million years ago, evolving three morphologically distinct forms within a life cycle (Erezyilmaz, 2006). The striking differences between the juveniles and the adults allow different habitats and food to be utilized at the respective stages, decreasing resource and spatial competition within species (Truman and Riddiford, 1999). Insects has been divided into three classifications based on the degree of morphological change they undergo postembryonically: ametaboly, hemimetaboly, and holometaboly. Ametabolous insects are wingless insects that undergo little to no metamorphosis (Erezyilmaz, 2006; Truman and Riddiford, 1999). The only difference is that the juvenile lacks functional sex organs (Erezyilmaz, 2006; Truman and Riddiford, 1999). Hemimetabolous insects undergo incomplete metamorphosis in which they begin as nymphs, their smaller adult form, that gradually develop wings and mature genitals through different molts (Erezyilmaz, 2006; Truman and Riddiford, 1999). In contrast, holometaboly, or complete metamorphosis, is drastically different from the previous two forms. Holometabolous insects undergo three distinct life history stages: the larva, the pupa, and the adult. It has been established that holometaboly most likely derived from hemimetaboly and ametaboly; how holometaboly evolved, however, is still a highly debated topic (Erezyilmaz, 2006; Truman and Riddiford, 1999).
The question of how complete metamorphosis evolved can be traced back to the 1600’s. Since then, the various arguments have condensed into two primary hypotheses put forth by Antonio Berlese and Howard E. Hinton. Berlese pioneered the idea of “de-embryonization”. According to this idea, the holometabolous larva is likened to an embryo capable of feeding and living independently of the egg (Erezyilmaz, 2006) (Fig 1). In essence, the larva is a result of premature hatching that is caused by a reduction of yolk in the egg (Truman and Riddiford, 1999). Because the larva is equivalent to an embryo according to this view, the pupa and the hemimetabolous nymph are by extension considered to be homologous phases (Fig 1). The nymphal instars are condensed into a single holometabous pupal stage (Erezyilmaz, 2006). The Berlese theory is further supported from findings by Truman and Riddiford. For hemimetabolous insects, Truman and Riddiford distinguish the early stage embryo from the “pronymph,” the embryonic stage immediately before the nymph stage (Truman and Riddiford, 1999). Like Berlese, Truman and Riddiford argued that the pronymph is the precursor of the holometabolous larva. Morphologically, the pronymph and larvae share many characteristics: both have soft and unsclerotized cuticles that differ from the nymphal and pupal stage respectively; the timing of cuticle secretion for each form is parallel to one another; and the sensory neurons found in both forms appear to be homologous units (Erezyilmaz, 2006). In contrast, Hinton argued that the larva is akin to the nymph stage and the pupa is a derivative of the final nymphal stage (Fig 1). His proposal is based on the development of adult muscles that arise during the pupal stage, making a transitional phase between the larval and adult. Here, the larva is not derivative of the embryo as there is evidence of larval structures that could not have originated from embryonic appendages (Truman and Riddiford, 2002).
Figure 1. Schematic diagram summarizing the Berlese and Hinton theories on the homology between hemimetabolous and holometabolous insects.

Berlese

Hinton
Juvenile hormone and hormonal regulation of metamorphosis

Endocrine studies looking at both hemimetabolous and holometabolous insects have provided further insight into the evolution of metamorphosis. Both hemimetabolous and holometabolous metamorphosis are regulated by ecdysteroids and juvenile hormone (JH) (Riddiford, 2007). JH, in particular, plays a key role in the shift from the larva to the adult, and from the pronymph to the nymph (Truman and Riddiford, 2002). Ecdysteroids are steroid hormones that induce molting, while JH regulates the characteristics of the molt (Riddiford, 2007). Ecdysteroids bind to a heterodimeric complex comprised of Ecdysone receptor/Ultraspireacle (EcR/USP) (Jindra, 2013). The bound complex activates a cascade of transcription factors, ultimately inducing molting (Jindra, 2013). During the larval and nymphal phase, the periodic elevation of ecdysone levels results in regular molting cycles (Truman and Riddiford, 2007). In hemimetabolous and holometabolous insects, molting results in the respective reformation of the nymphal and larval bodies; the main difference being a change in isometric growth (Truman and Riddiford, 2007). During the juvenile phase, JH is crucial in preventing precocious metamorphosis by modifying the animal’s response to ecdysone. Ectopic application of JH during the final instar typically leads to supernumerary molts, increasing the size of the nymph or larva while inhibiting metamorphosis (Truman and Riddiford, 2007). In the absence of JH, ecdysteroids induce metamorphosis, while the presence of JH keeps the animal in its juvenile phase as it molts (Riddiford, 2007). In holometabolous insects, JH is present throughout the larval stage until the final instar when a drop in JH levels initiates a surge of ecdysone that results in pupal commitment (Truman and Riddiford, 2002). JH re-emerges in concert with another peak of ecdysone for the pupal molt before it disappears, preventing precocious adult development (Truman and Riddiford, 2002). In hemimetabolous insects, the
process is similar; a decrease in JH levels allows the nymph to progress to an adult (Truman and Riddiford, 2002).

Although JH plays such a crucial role, the nature of its signaling is still an enigma. Recently, several factors downstream of JH have been identified. JH signals through the Methoprene-tolerant (Met) receptor (Konopova et. al., 2008). Studies on Tribolium castaneum show that the loss of Met results in precocious pupation (Konopova et. al., 2008). Met also regulates the expression of transcription factor Krüppel-homolog 1 (Kr-h1) and Broad-Complex (BR-C) (Konopova et. al., 2011). In hemimetabolous and holometabolous insects, Krh1 acts as a JH-induced repressor of adult morphogenesis. In contrast, BR-C acts differently in the two classes; in holometabolous metamorphosis, BR-C is required for specifying pupal features while in the hemimetabolan insect order, BR-C expression was not linked to metamorphosis (Konopova et. al., 2011; Zhou and Riddiford, 2002, 2001, 2004). Suzuki et. al. found that suppression of the Broad gene (br) results in the formation of individuals that had both larval and adult traits, but no pupal traits, indicating that br is crucial in pupal development (Suzuki et. al., 2008). The evolution of br function suggests that br played a key role in the evolution of the specialized pupal morphology (Suzuki et. al., 2008). Although progress has been made in the identification and role of the JH response genes, such as Kr-h1 and BR-C, the specific JH target genes have remained elusive.

**Imaginal cells in holometabolous insects**

A critical feature of holometaboly and the morphogenesis of the adult structure from the juvenile lies in the existence of imaginal cells and imaginal discs. Imaginal cells are precursors to the pupal and adult cell populations (Krasnow, 2008). These cells are absent from
hemimetabolus insects and therefore are likely to be holometabolous innovations. Understanding the developmental regulation of imaginal cells may shed light on the process by which holometaboly may have evolved. In Drosophila, the imaginal cells are typically clustered as discs and defined as epithelium detached from the cuticle (Truman and Riddiford, 2002). The adult external appendages, legs, wings, and antennae, arise from their respective imaginal discs, which proliferate and differentiate (Kojima, 2004). The discs proliferate as the larva progresses through multiple instars but provide no functional or structural contribution to the larval body. In Drosophila, the imaginal discs proliferate in a concentric and folded manner while remaining as a monolayered- epithelium during the larval stage (Kojima, 2004). During the pupal stage, the disc elongates and the cells rearrange themselves to form the corresponding adult structure (Kojima, 2004).

In other holometabolous insects, the larval and imaginal cells contribute to the adult structures in several different ways. In one case, the animal lacks imaginal cells, and polymorphic larval cells pattern all of the adult structures. Tenebrionid beetle legs and antennae, for example, have been suggested to derive from polymorphic cells (Nagel, 1934; Truman and Riddiford, 2002). In other cases, proliferation of the imaginal discs begins with the apoptosis and degeneration of larval cells that are specialized for larval function or are unnecessary in adult morphology as seen in Manduca sexta. In Manduca, the adult appendages derive primarily from imaginal cells (Tanaka and Truman, 2005).

The molecular cascade governing the proliferation and function of imaginal discs is still under investigation. The development of Drosophila leg imaginal disc has been widely studied and provides information on the genetic components underlying disc-derived patterning. In Drosophila, each subsequent region of the leg imaginal disc after each instar appears to be
controlled by a series of transcription factors (TFs) responsible for proximal-distal development (Kojima, 2004). Expression profiles indicate that imaginal disc proliferation is controlled by a core group of transcription factors: *Distal-less (Dll), homothorax (hth), dachshund (dac)* (Kojima, 2004). These transcription factors are expressed in both the proximal and distal regions of the proliferating disc and in turn, they induce the expression of other genes (Kojima, 2004). The expression of *Dll, hth*, and *dac* are confined to specific regions (Kojima, 2004). Studies show that the boundaries and spatial distribution of the TFs are established by secreted proteins called morphogens that act upstream of the TFs (Kojima, 2004). Varying concentrations of the morphogens throughout the disc dictate the placement and expression level of the TFs. Several morphogens identified are Wingless (Wg), Decapentaplegic (Dpp), Hedgehog (Hh), and the Epidermal growth factors (EGFs) (Kojima 2004). Hh is at the top of the cascade; its secretion induces Wg and Dpp, which in turn activates TFs that regulate imaginal disc proliferation and patterning (Kojima, 2004).

JH has also been shown to regulate the proliferation of imaginal cells. However, despite our knowledge of the mechanism governing development and imaginal discs, it is still unclear which of these morphogens is actually a target gene of JH. Our lab has found through a comparison of the transcriptomes of larval legs undergoing larval-to-larval molt and prepupal legs that *hh* expression is decreased during the larval-larval molts relative to prepupal stage. This suggests a potential relationship between JH and Hh, and since Hh is at the top of the regulatory cascade of imaginal disc growth and proliferation, Hh is an interesting candidate to study in terms of understanding the origins of imaginal cells.
*Hedgehog Signaling Pathway*

The Hh pathway is highly conserved and has deep evolutionary roots. Hh is a secreted protein that acts as a morphogen. Hh is involved in development in both vertebrates and invertebrates. In vertebrates, the Hh homolog, Sonic hedgehog (Shh) is necessary during embryonic limb development. In both chick and mice limbs, Shh is involved in the posterior zone of polarizing activity and the maintenance of the apical ectodermal ridge (Phillip, 2011). Likewise, in *Xenopus*, Shh is needed for anterior-posterior axis patterning in limb development (Phillip, 2011). As mentioned previously, Hh signaling plays a key role in holometabolous insect metamorphosis. In the *Drosophila* larval limbs, Hh is expressed in the posterior compartment and diffuses into the anterior compartment. Its diffusion induces Dpp expression in the anterior-dorsal cells and Wg in the anterior-ventral cells (Basler, 1994). In the presence of the Hh ligand, the transmembrane protein Smoothed (Smo) translocates from a vesicle within the cytoplasm to the plasma membrane (Phillip, 2011). Smo is then phosphorylated by a complex consisting of three proteins, glycogen synthase kinase 3 (Gsk3), protein kinase A (Pka), and casein kinase 1 (Ck1). In turn, the phosphorylation of Smo allows the scaffold protein Costal-2 (Cos2) and the suppressor of fused (Sufu) to be phosphorylated by Fused (Fu). Consequently, the transcription factor, Cubitus interruptus (Ci) is able to separate itself from Sufu and Cos2, translocating into the nucleus where it activates the transcription of Hh target genes. In the absence of the Hh ligand, the Hh receptor Patched (Ptc) acts as an inhibitor of Smo. The inhibition of Smo prevents its migration into the plasma membrane, resulting in Cos2 forming a signaling complex composed of multiple serine and threonine kinases. This complex recruits Ci where it is then phosphorylated by Gsk3, Pka, and Ck1. Phosphorylation of Ci activates the protein Slimb (Slmb) where it catalyzes the ubiquitylation of Ci, marking Ci for degradation by a proteasome. As such,
Cubitus Interuptus (Ci) acts as a repressor transcription factor, preventing the transcription of Hh target genes (Fig. 2) (Phillip, 2011).

**Figure 2.** Schematic of Hedgehog (Hh) Signaling Pathway. (A) In the presence of the Hh ligand, a series of phosphorylations result in the transcription factor, Cubitus Interuptus (Ci), acting as an enhancer and promoting the transcription of Hh target genes. (B) In the absence of the ligand, the Hh receptor, Patched (Ptc), leads Costal-2 (Cos2) to form a complex that signals the degradation of Ci. In its cleaved form, Ci acts as a repressor and inhibits the transcription of Hh target genes.
A notable feature of the Hh pathway is its role in regeneration. Vertebrate limb regeneration is characterized by a series of steps: wound closure, blastema formation, and limb repatterning (Endo, 2004). Ablated limbs are covered by epidermal cells and after closure, undifferentiated cells capable of regeneration accumulate at the wound site, resulting in an outgrowth called a blastema (Endo, 2004). As cell proliferation continues, the blastema elongates. The accumulated cells then differentiate to become specialized tissue (skeletal, muscular, dermal, connective, etc.) necessary for limb reformation (Tanaka, 2003). Although a variety of signals are needed for initiating and completing regeneration, the Hh signaling pathway appears to play a crucial role. In Xenopus, Shh stimulates chondrogenesis in froglet limbs (Yakushiji, 2009). In ablated limbs, activation of Shh signaling increases cell proliferation resulting in a limb blastema and tissue differentiation (Yakushiji, 2009). In mammalian models, Shh is activated in injured mice liver and promotes the growth of liver progenitor cells and fibrogenic repair (Ochoa, 2010). Interestingly, the process of limb regeneration in vertebrates and insects share many similarities. As with vertebrate limb regeneration, the same steps apply. Immediately following ablation, epidermal cells migrate across the wound site to form a scab (Mito, 2002). Cell proliferation beneath the scab results in thickening of the epidermis to form the blastema (Mito, 2002). The limb is then restored with the differentiation of the cells beneath the epidermis to form a fully functional and developed limb (Mito, 2002). Interestingly, the role of Hh signaling appears to be somewhat different between hemimetabolous insects and holometabolous insects. In crickets, a hemimetabolous insect, when hh is removed, a branched leg regenerates (Nakamura et al, 2008). However, knockdown of hh in the beetle Tribolium castaneum results in lack of regeneration of limbs after blastema formation, indicating that hh is necessary for limb regrowth (Villareal, 2013). Similarly, crickets do not show any defects when
hh is removed from the embryonic stage (Nakamura et al., 2008; Miyawaki et al., 2004). In contrast, in Tribolium, removal of hh expression during embryogenesis leads to truncation and deformation of limbs appendages (Farzana and Brown, 2008). In addition, removal of Patched, the antagonist of Hh signaling, during embryogenesis leads to enlarged appendages (Farzana and Brown, 2008). Thus, there are a few differences in the functions of Hh between hemimetabolous insects and holometabolous insects. How this signaling pathway functions during postembryonic development should provide interesting insights into the regulation and evolution of imaginal cells.

*Tribolium castaneum*

In our study, the red flour beetle, *Tribolium castaneum*, was used to determine the role of Hh signaling in cell proliferation during metamorphosis. A member of the order Coleoptera, *Tribolium* is a holometabolous insect (Park 1934). Like other holometabolous insects, its life cycle is characterized by three distinct stages: the larva, pupa, and adult. As a larva, *Tribolium* typically undergoes seven to eight instars before proceeding to the pupal phase; its full life cycle is approximately 30 days (Park 1934). Its short life-span and high productivity rate allows multiple experiments and trials to be conducted within a short time. In terms of diet, *Tribolium* requires only flour, making maintenance relatively easy (Park 1934). The phylogenetic placement of Coleoptera order also makes *Tribolium* an ideal model system for our investigation. Recent studies show that insects in the Coleoptera and Hymenoptera orders represent orders of the holometabolous insects that branched off relatively early after the evolution of holometabolism. As previously mentioned, there are three primary modes of limb development, depending on the nature of larval and imaginal cell proliferation. It is thought that the use of polymorphic larval
cells, instead of imaginal discs, is the basal condition. The phylogenetic position of *Tribolium* with its primitive mode of limb development allows us to investigate the transition from hemimetaboly to holometaboly.

**RNA interference**

Our understanding of various structures and functions in biological systems has become increasingly more comprehensive with new tools and technologies. RNA interference (RNAi) has become a widely used tool in analyzing gene function. RNAi is a biological process used as a molecular immune response against viruses and foreign RNAs. The introduction of double-stranded RNA (dsRNA) initiates the formation of a complex that degrades the dsRNA as well as any other RNAs, single or double, with an identical coding sequence. This response was first experimentally utilized in *Caenorhabditis elegans* where the insertion of dsRNA coding for a *C. elegans* gene resulted in a phenotypic abnormality in the animal (Fire et al., 1998). Experimentally, since the inserted dsRNA has the same sequence as an endogenous gene, the animal not only degrades the dsRNA but also the messenger RNA (mRNA) with the same coding sequence. This prevents the protein product of the gene from being produced, and essentially silences the gene function in the animal. To this end, RNAi provides information on gene function as well the phenotypic impact due to the gene knockdown.

RNAi has been shown to be a very versatile tool. Because dsRNA can travel systemically throughout the body from any origin, administration of dsRNA can occur in various ways, via injection, oral intake, or ectopic application (Fortunato and Fraser, 2005). However, despite refinements in the process, RNAi has only been successful in a few animals and not all genes appear to be susceptible to RNAi (Simmer, et al., 2002). Fortunately, previous studies
have shown that RNAi experiments done on Tribolium larvae are successful and function systemically (Tomoyasu and Denell, 2004).

**Hedgehog signaling in appendage remodeling during metamorphosis**

In this present study, we will examine the roles of Hh signaling in Tribolium. In particular, we will be addressing these main questions: What are the roles of Hh and Ptc during metamorphosis? How does JH regulate the transition between larval to adult structures? Is there an interaction between JH and the Hh pathway? Although it is clear that Hh signaling is needed, the specific effects on cell proliferation have yet to be explored. Given that Ptc works antagonistically with hh, looking at both would provide further insight into their interaction and their phenotypic effects. The JH regulatory system is poorly understood. It has no known homologue in vertebrates, and it is a sesquiterpenoid lipid, making it difficult to study. One unknown factor is whether JH has a target gene and if so what is it. The heavy involvement of Hh in development and the aforementioned transcriptome results point towards Hh signaling as a possible JH target. Our study has implications in understanding the evolution of holometabolism, JH regulatory mechanism as well as Hh as a conserved regulator between invertebrates and vertebrates. Based on our preliminary findings and on what is known about JH and Hh, we hypothesized that Hh plays a crucial role in Tribolium development, particularly in cell proliferation. As JH inhibits growth, we also hypothesized that JH and Hh interact, either directly or indirectly.
MATERIALS AND METHODS

Beetle husbandry

Wildtype *Tribolium castaneum* strain GA1 was provided by Dr. Richard Beeman (USDA ARS Biological Research Unit, Grain Marketing & Production Research Center, Manhattan, Kansas). All beetles were raised on organic whole wheat flour containing 5% nutritional yeast and incubated at 29°C and 50% relative humidity.

Double stranded RNA synthesis

In order to perform RNA interference on the animals, double stranded RNA (dsRNA) was made. *Tribolium* at various larval stages and sizes were dissected in 1X-phosphate buffer saline (PBS; 0.02 M phosphate, 0.15 M NaCl, 0.0038 M NaH$_2$PO$_4$, 0.0162 M Na$_2$HPO$_4$; pH 7.4). The gut was removed while the remaining tissue was homogenized in TRIzol (Invitrogen). RNA was isolated by treating the homogenized mixture in chloroform, and RQ1 RNase-Free DNase (Promega) was used according to the manufacturer’s instructions to purify the isolated RNA sample. One μg of RNA was converted to cDNA through the process of reverse transcription using Fermentas First Strand cDNA Synthesis Kit (Thermo Scientific, MA USA) as instructed by the manufacturer.

Using the synthesized cDNA, the desired sequences (*hh, ptc, and cos2*) were amplified via Polymerase Chain Reaction (PCR). *hh* (GenBank accession number NM_001114365), *ptc* (GenBank accession number NM_001134377), and *cos2* sequences (GenBank accession number XM_968298) were obtained from Genbank. Primers were designed (Table 1) using Primer3 amplified using GoTaq® PCR Core System I (Promega, WI USA). PCR products were analyzed by agarose gel electrophoresis and then extracted using MinElute Gel Extraction Kit (Qiagen,
USA) according to the manufacturer’s instructions. Extracted amplified cDNA was then inserted into a TOPO TA vector using TOPO® TA Cloning Kit for Sequencing (Invitrogen) and transformed into chemically competent *E.coli* cells.

**Table 1. Primers used in this study**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>hh</em></td>
<td>Forward</td>
<td>CCTCTCCTCGCTCCAAATC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CAAAGGTCTATCCGCACTACC</td>
</tr>
<tr>
<td><em>ptc</em></td>
<td>Forward</td>
<td>GCGGTGCCAATAAGAATAAA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GTACGGTTGTGGCCTTCTC</td>
</tr>
<tr>
<td><em>cos2</em></td>
<td>Forward</td>
<td>GGAAAGTCCTACACGCTCCT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ATATGCACGCACCAATTCC</td>
</tr>
<tr>
<td><em>hh</em></td>
<td>(knockdown verification)</td>
<td>Forward</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
</tr>
<tr>
<td><em>ptc</em></td>
<td>(knockdown verification)</td>
<td>Forward</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
</tr>
<tr>
<td><em>cos2</em></td>
<td>(knockdown verification)</td>
<td>Forward</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
</tr>
<tr>
<td><em>hh</em></td>
<td>(qPCR)</td>
<td>Forward</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
</tr>
<tr>
<td><em>ptc</em></td>
<td>(qPCR)</td>
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<tr>
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<td></td>
<td>Reverse</td>
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<tr>
<td><em>dpp</em></td>
<td>(qPCR)</td>
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<tr>
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<td>(qPCR)</td>
<td>Forward</td>
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<td></td>
<td></td>
<td>Reverse</td>
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</tbody>
</table>
Plasmid DNA from the cloned vector was purified using QIAprep Spin Miniprep Kit (Qiagen, USA) and then linearized through a restriction digest by restriction enzymes \textit{SpeI} and \textit{NotI} (NE Biolabs, Ipswich, MA) and treated with phenol chloroform for purification. Single-stranded RNA (ssRNA) was synthesized using 1 μg of plasmid digest and MEGAscript T3 and T7 kits (Life Technologies, CA USA). Equal concentrations of complimentary strands of ssRNA were annealed to make dsRNA using a thermal cycler. dsRNA synthesis was performed as described by Hughes and Kaufman (2000). Proper annealing was verified by visualizing both dsRNA and ssRNA on an agarose gel. dsRNA was stored at -80°C.

\textbf{dsRNA injections}

Approximately 0.5 μg of either \textit{hh}, \textit{cos2}, or \textit{ptc} dsRNA was injected into either day zero seventh or sixth instar larvae. Larvae were anesthetized by placing the animals on ice. Injections were done using pulled-glass capillary tubes where the needle containing dsRNA was inserted between the first and second abdominal segments on the dorsal side of the larva. Control animals were injected with the same amount of bacterial \textit{ampicillin resistance (amp$^r$)} dsRNA (plasmid obtained from Dr. Takashi Koyoma, the Gulbenkian Institute of Science, Portugal). After injections, animals were separated into individual plastic containers with whole wheat flour and maintained under normal conditions. For \textit{cos2} and \textit{ptc} dsRNA-injected animals, phenotypes were observed in three-four day increments after injections. For \textit{hh} animals, phenotypes were observed in four days increments and daily after one larval molt in order to use them for BrdU assays on day zero of the prepupal stage.
**BrdU staining of proliferating cells.**

In order to see the effect of *hh* knockdown on cell proliferation, BrdU cell proliferation staining assay was conducted on *hh* dsRNA-injected animals. Day zero prepupae were anesthetized and then dissected in 1X-PBS solution. The ventral section containing the larval limbs were dissected and incubated in a solution of 20 μg/μL of BrdU at room temperature for 3 hours. The dissected portion was fixed in a solution of 3.7% formaldehyde in 1-X PBS. Within an hour of formaldehyde fixation, the tissue was placed back in PBS solution where the leg tissues were dissected out and placed back in the formaldehyde solution. After an overnight fixation, the tissue was washed with a solution of PBS with 1% Triton-X 100 (PBS-TX). The tissue was then submerged in a solution of 2 N HCl in PBS-TX for one hour at 37ºC. After treatment, the acid solution was removed and the tissue was rinsed in PBS-TX. Tissues were then blocked with 5% NGS in PBS-TX for 30 min. at room temperature. Post-blocking, tissues were incubated in 1:200 anti-BrdU antibody in PBS-TX overnight at 4ºC. Subsequently, the anti-BrdU antibody was removed and the tissue was rinsed again. The tissue was then incubated in a solution of 1:1000 Alexa Fluor 488 goat anti-mouse antibody in PBS-TX overnight at 4ºC. The secondary antibody was removed and after several washes in PBS-TX, the tissue was mounted in either Vectashield or 100% glycerol. Mounted tissues were imaged using the Nikon Eclipse 80i fluorescence microscope, and images were taken using a QImaging camera (Diagnostic Instruments) and NIS Elements Imaging Software. Images were stacked using Image J (NIH Image).

**Quantitative PCR (qPCR)**

Expression profile of *hh* and *ptc* in the presence and absence of juvenile hormone (JH) during metamorphosis was analyzed using qPCR. A JH analogue, Methoprene (30 μg/μl in
acetone), was ectopically applied, using a micropipette, to the dorsal side of day zero final instar larvae. For control larvae, acetone, the solvent used for methoprene treatments, was applied. The treated animals were collected daily until day four and their RNA was isolated as described above. RNA was converted to cDNA as described above and mixed with SYBR Green Supermix and qPCR primers of either hh, ptc, or Ribosomal protein 49 (rp49) (1.0 μL cDNA whole body larvae, 0.5 μL forward primer, 0.5 μL reverse primer, 10 μL SYBR Green PCR Master Mix and 8.0μL deionized water). Each sample underwent 50 cycles at 60°C. Three biological replicates of each treatment were made and analyzed to ensure the reproducibility of the data. Each biological sample was assayed in triplicate.

**Knockdown verification**

Downregulation of ptc and cos2 mRNA expression in dsRNA treated animals were confirmed using semi-quantitative reverse transcription-PCR (RT-PCR). hh knockdown verification was previously done (Villareal, 2013). Day zero seventh instar larvae were injected with either cos2 or ptc dsRNA. Control animals were injected with amp’ dsRNA. RNA was isolated from the dsRNA-treated animals on day zero of the eighth instar. cDNA was synthesized from 1 μg of the isolated RNA. The amount of mRNA expression in the samples was quantified using RT-PCR. rp49 again was used as a control to ensure that equal amounts of cDNA were used. Each sample underwent thirty-five cycles.

**RESULTS**

**Knockdown of ptc and cos2 expression results in ectopic appendage outgrowths**

In order to characterize the role of Ptc and Cos2 in Tribolium development, ptc or cos2 dsRNA was injected into day zero seventh and sixth instar larvae. Of the larvae that survived post
injection on day zero sixth and seventh instars, 53 out of 102 ptc dsRNA injected larvae or 28 out of 46 larvae injected with \textit{cos2} dsRNA as sixth instars or 23 out of 41 larvae injected with \textit{cos2} dsRNA as seventh instars underwent an additional one or two larval molts. With the exception of a few larvae, 87\% of all \textit{ptc} dsRNA injected animals and 98\% of all \textit{cos2} dsRNA injected animals showed a defective phenotype within the first two molts post injection (\textit{ptc}: n=53; \textit{cos2}: n=51; Table 2).

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>Leg &amp; antennae</th>
<th>Leg</th>
<th>Combination</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{ptc}</td>
<td>102</td>
<td>6</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Died prior to first molt</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survived past first molt</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>\textit{cos2}</td>
<td>46</td>
<td>6</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>\textit{amp r}</td>
<td>41</td>
<td>1</td>
<td>0</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 2. Phenotypes found in \textit{ptc} and \textit{cos2} dsRNA treated animals

\begin{tabular}{|c|c|c|c|c|}
\hline
Phenotypes & Leg & antennae & Leg & Combination & Normal \\
\hline
\textit{ptc} & 102 & 6 & 4 & 7 & \\
\textit{cos2} & 46 & 6 & 2 & 8 & \\
\textit{amp r} & 41 & 1 & 0 & 10 & \\
\hline
Died prior to first molt & | | | | \\
Survived past first molt & | | | | \\
\hline
\end{tabular}
Knockdown of either *ptc* or *cos2* expression caused alterations to the larval limb and head morphology (Figs. 3 and 4). The comparable phenotypes observed suggest that both *cos2* and *ptc* knockdowns disrupt the Hh signaling pathway in similar ways. Limb morphology was most commonly altered in these knockdown animals (*ptc*: n=36/53; *cos2*: n=36/51; Table 2). The legs were affected to varying degrees but in all cases, outgrowths were apparent on the legs. These outgrowths varied in position, and were found along the femur, tibia, and tarsus. Depending on the size and nature of the outgrowth, the knockdown led to legs with small bumps or dramatically thickened legs (*ptc*: n=19/53; *cos2*: n=17/51; Fig. 3B, C). In more severely affected legs, the legs segments disappeared (Fig. 3B). Duplication of the claw was also seen in some animals (*ptc*: n=4/53; *cos2*: n=7/51; Fig. 3C). In normal development, *Tribolium* larvae only have a single claw on each leg; two clawed legs are only seen in the adult morphology. In the treated larvae, duplicated claw developed. In addition, some legs had sclerotized teeth-like projections along the proximo-distal axis (Fig. 3C, arrows).

Morphological changes were also seen in the larval head and body of *ptc* knockdown larvae. Larval antennal morphology was severely modified by silencing either *ptc* or *cos2* expression. In some animals, the normally smooth, and straight segmented antennae became curved and thick (*ptc*: n=12/53; *cos2*: n=20/51; Fig. 4B,E,H). In other larvae, the antennae appeared flattened and spherical (*ptc*: n=1/53; *cos2*: n=2/51; Fig. 4C,F,K). In addition, ectopic head bumps were also seen in *ptc* and *cos2* knockdown animals (*ptc*: n=4/53; *cos2*: n=7/51; Fig. 4I, L). In all *ptc* and *cos2* knockdown animals with two outgrowths, the bumps were either associated with the antennae (Fig. 4) or appeared as ectopic outgrowths from the head cuticle (Fig. 4); other head outgrowths formed at the center of the head (Fig. 4I, L). In addition, *ptc* and *cos2* knockdown larvae had morphological changes on the larval body. In the anterior second
and third thorax segments, protrusions were observed (Fig. 4C). As these segments are where the wing buds emerge from, we suspect that these outgrowths may be premature wing buds.
Figure 4. Effect of ptc and cos2 on the larval head and body phenotype of Tribolium. (A,D,G,J) Examples of ninth instar control larvae injected with 2 μg/μL amp' dsRNA on day zero seventh instar. (B,E,H) Ninth instar larvae injected with 0.5 μg/μL ptc dsRNA on day zero seventh instar. Arrows show head bumps (E, I, L), the formation of the compound eye (H), modified antennae (K, H), and wing budding (C). Ninth instar larve injected with 2 μg/μL cos2 dsRNA on day zero seventh instar. Scale shown is 0.5mm.
Figure 5. Effect of ptc knockdown on the pupal and adult phenotypes of Tribolium. (A-C) Control pupa injected with 2 μg/μL amp’ dsRNA on day zero seventh instar larva. This animal pupated after 1 molts post dsRNA injection. (D) Pupa injected with 0.5 μg/μL ptc dsRNA on day zero seventh instar. This animal pupated after one molt post dsRNA injection. (E-F) Pupa injected with 0.5 μg/μL ptc dsRNA on day zero seventh instar. Animal pupated after one molt post dsRNA injection. (G-I) Adult injected with 2 μg/μL amp’ dsRNA on day zero seventh instar larva. (J-M) Adult injected with 2 μg/μL ptc dsRNA on day zero seventh instar. Adult is the same animal as in pupa seen in (E-F). M represents an animal whose pupal cuticle was removed. Arrows show the formation of the compound eye (E, F, K, M), modified antennae (L), and ectopic head appendage (J, E). Scale bars represent 0.5mm.
In *ptc* knockdowns, two animals survived through the pupa stage to the adult (n= 2/18 pupae; Fig. 5). In one pupa, the wings were malformed, and several of the pupal legs appeared thicker (Fig. 5D). In another pupa, three ectopic head outgrowths were seen. The two more lateral growths had the appearance of compound eyes, while the center head bump looked like an appendage (Fig. 5E, F). The phenotypes seen in the pupa persisted to the adult stage where they developed into adult compound eyes and an antenna-like appendage (Figure 5J-M). The antenna-like appendage grew out of a socket, indicating that this appendage had joints at the base (Fig.5M). In addition, one of the adult antennae was forked and had duplicated distal segments (Fig. 5L). The persistence of the phenotypes from the larva to the adult stage indicates that the knockdown effect of *ptc* carries through the different developmental stages.

**Prepupal cell proliferation decreases in the absence of Hh.**

*hh* expression was silenced so that the role of Hh signaling during prepupal leg development could be examined. *hh* dsRNA was injected into day zero seventh instar larvae, and early prepupa legs were isolated for cell proliferation assay. *hh* RNAi animals had diminished levels of cell proliferation compared to the prepupal legs isolated from *amp′* dsRNA-injected animals (Fig. 6). In particular, distinct regions of the leg showed an absence of cell proliferation in the tibia and femur, while the proximal tibial region appeared to have proliferated to a similar degree as our control animals (*hh*: n=9; *amp′*: n=4, Fig.6B, C). In addition, the tarsus contained a small region of increased proliferation (Fig. 6B, C) This regionalized cell proliferation suggests that distinct cell populations proliferate under the control of different cues and that Hh regulates two major regions of the leg.
Figure 6. Effect of \( hh \) knockdown on Tribolium prepupal leg cell proliferation.

A) Control prepupal leg injected with 2 μg/μL \( ampu \) dsRNA on day zero sixth instar larva. 
The prepupa developed after 2 molts post dsRNA injection. 

B) Prepupal leg injected with 2 μg/μL \( hh \) dsRNA on day zero sixth instar larva. 
The prepupa developed after 2 molts post dsRNA injection. 

C) Shows the same leg as B with the colors inverted, and the localized regions lacking cell 
proliferation highlighted in yellow to emphasize the contrast. BrdU cell proliferation assay were 
done on these animals using the protocol described in Methods and Materials. Scale bars represent 
0.5 mm.
**Hh expression is low when JH levels are high and increases during the onset of metamorphosis**

In tenebrionid beetle larvae, the final instar JH levels are initially high; subsequent decrease in JH signals the larval tissues to commit to pupal fates (Quennedey and Quennedey, 1999). When methoprene was topically applied to day 0 seventh instar *Tribolium* larvae, they underwent a supernumerary molt (Table 3). In contrast, acetone-treated animals initiated prepupation on day five of the seventh instar (Table 3). A quantitative PCR analysis of the *hh* and *ptc* expression during the larval and prepupal stages showed that animals treated with methoprene as day zero seventh instar and collected on day four has a significantly lower level of *hh* expression than those treated with acetone (p=0.03; Fig. 7A). There were no statistical differences in *ptc* expression between the acetone and methoprene treated larvae on any day.

| Table 3. Comparison of Methoprene and Acetone treatments on *Tribolium* larva |
|---------------------------------|------------------|------------------|
| Treatment                      | Supernumary Larval Molts (n) | Pupation (n)    |
| Methoprene                     | 6                              | 0                |
| Acetone                         | 0                              | 6                |

As *hh* expression appeared to be affected by the presence of JH, we suspected a possible interaction between JH and the Hh signaling pathway. To determine whether JH might influence Hh directly, expression of *hh* and two of its target genes, *decapentaplegic (dpp)* and *wingless (wg)*, were examined 16 hrs after JH was applied. Methoprene was applied on day 4 seventh instar larvae and collected 16 hours later. qPCR analyses showed that the *hh, dpp*, and *wg* expressions of methoprene-treated animals and acetone (control) animals were not significantly different from each other (Fig. 8). Thus, while JH application can inhibit increase of *hh* expression when applied early in the instar, it does not appear to influence Hh expression immediately on day 4 of the final instar larva.
Figure 7. Effect of Methoprene (JH analogue) on the expression profile of hh and ptc in Tribolium larvae. Methoprene or acetone (control) was applied to day zero seventh instar and collected on day 1-4. qPCR analysis was done as described in Methods and Materials A) qPCR analysis of hh expression on day 1-4 of the penultimate larval instar. B) qPCR analysis of ptc expression on day 1-4 of the penultimate larval instar. Statistical analysis was done on JMP. * Represents statistically significant difference. Error bars represent standard error.
Confirmation of gene knockdowns

A semi-quantitative RT-PCR was performed for \(hh\), \(ptc\), and \(cos2\) to confirm that the dsRNA injections resulted in the knockdown of the corresponding gene. Day zero seventh instar larvae were injected with \(amp\)' and either \(hh\), \(ptc\), or \(cos2\) dsRNA. cDNA were collected on day zero of the eight instar. RT-PCR showed that the expression of \(hh\), \(ptc\) and \(cos2\) were knocked down in the \(hh\), \(ptc\) and \(cos2\) dsRNA-injected larvae, respectively, relative to the \(amp\)' dsRNA injected larvae (Fig. 9).

Figure 9: Knockdown verifications of dsRNA injected larvae. Day zero seventh instar larvae were injected with either 2 \(\mu\)g/\(\mu\)L \(amp\)', \(cos2\), or 0.5 \(\mu\)g/\(\mu\)L of \(ptc\) dsRNA and cDNA was synthesized from pre-pupae. The cycle number for \(amp\)', \(cos2\), and \(ptc\) was 35 cycles and rp49 was 34 cycles. \(hh\) knockdown verification was previously confirmed (Villareal, 2013).

Figure 8. Effect of Methoprene (JH analogue) on the expression profile of \(dpp\), \(wg\), and \(hh\) in \textit{Tribolium larvae}. Methoprene or acetone (control) was applied to day 4 seventh instar and collected 16 hours later. qPCR analysis was done as described in Methods and Materials. Statistical analysis was done on JMP. Error bars represent standard error.
DISCUSSION

In this study, the role of the Hh signaling pathway during metamorphosis in *Tribolium castaneum* was examined. Specifically, the phenotypes that emerge due to knockdown of *hh, ptc,* or *cos2* were investigated using RNA interference. In both *ptc* and *cos2* knockdown larvae, prominent tissue overgrowth was observed leading to disrupted patterning of pre-existing appendages, and ectopic head and body outgrowths in the larva. Cell proliferation assays on prepupal legs showed that the absence of *hh* prevented cell proliferation in localized regions. The low *hh* expression in methoprene-treated larvae also implied a possible interaction with JH. Together, our data show that Hh may be a key regulator of allometric appendage growth and that JH may indirectly regulate Hh expression.

**Hh signaling pathway is necessary for regulating cell proliferation during larval and adult development**

BrdU assay indicated that *hh* knockdown leads to diminished cell proliferation in the prepupal legs. Previous work done in the lab has shown that Hh is also essential for larval appendage regeneration (Villareal, 2013). Thus, both larval leg regeneration and adult leg development appear to rely on Hh. Assays show localized regions of proliferation and diminished cell proliferation. The localized loss of cell proliferation in the prepupal legs suggested that there are two distinct populations of cells: one that is under the control of Hh and another whose proliferation is independent of Hh.

As with *Tribolium,* Hh plays a key role in *Drosophila* limb development (Kojima, 2004) and regulates imaginal disc cell proliferation (Duman-Scheel et al., 2008). Furthermore, the *Drosophila* leg imaginal disc is composed of two sets of cell populations, one of which is directed by Hh (Kojima, 2004). The two cell populations are divided based on the anterior-
posterior compartments (Kojima, 2004). Hh is initially expressed in the posterior region and diffuses into the anterior compartment (Kojima, 2004). Based on the concentration of Hh, other genes are activated (Kojima, 2004). In contrast to Drosophila, we did not see a posterior versus anterior division in cell populations, but rather, the middle of each segment appeared to be controlled by HH. Therefore, the area of Hh activity is spatially different between Tribolium and Drosophila, but Hh has similar roles in Drosophila and Tribolium leg cell proliferation.

Since Hh acts antagonistically with Ptc and Cos-2, knockdown of Ptc and Cos-2 leads to overexpression of Hh target genes. Ectopic structures, particularly on the appendages and head were seen when Ptc or Cos-2 expression was silenced. The similarity in phenotypes resulting from either Ptc or Cos-2 knockdown suggests that the phenotypic effects observed are due to an overactivation of Hh signaling in both cases. In Drosophila, localized upregulation of Hh or downregulation of Ptc results in increased cell proliferation in the wing imaginal disc (Duman-Scheel et al., 2008; Johnson et al., 2000). The cell overproliferation seen in Drosophila is consistent with the tissue overgrowth seen in Tribolium. Thus, overexpression of Hh appears to result in cell overproliferation in both Tribolium and Drosophila. However, it is unclear how the cell overproliferation in Drosophila imaginal discs would translate in the adult stage since to our knowledge, there have been no studies that have looked at the systemic effects of Ptc and Costal mutations on the postembryonic development of the fly. Interestingly, in addition to appendage overgrowth, some of the larval phenotypes obtained from silencing ptc and cos2 bear striking resemblance to the morphological changes that manifest during metamorphosis. These include the claw duplication, the development of wing-like protrusions, and the formation of compound eyes. Thus, we suggest that overexpression of Hh in the larva might lead to premature maturation of appendage structures.
Our data indicate that suppression of Hh is necessary for proper maintenance of larval appendages, whereas activation of Hh is required for appendage remodeling during metamorphosis. Our qPCR results showing that hh expression is low during the larval stage and rises as the larvae enter the prepupal stage support this view. The phenotypes observed from ptc and cos2 knockdown together with the qPCR results suggest that Hh is being actively suppressed, potentially by JH, a key regulator of metamorphosis.

**Interaction between Hh and JH**

One of the major gaps in our understanding of metamorphosis is the link between endocrine regulators and patterning. Studies in *Tribolium* as well as other insects show that JH acts as a status quo hormone that also has a morphostatic function, preventing imaginal cells from proliferating (Truman et al., 2006, 2007). However, the mechanisms by which JH inhibits imaginal cell proliferation remains unknown. Our findings indicate that Hh is a key regulator of cell proliferation in metamorphosizing appendages and that its activity must be inhibited during the earlier larval stages. Because JH promotes isometric growth during the larval stage, appendage development overall may be controlled by the interaction between Hh and JH. As previously mentioned, Hh appears to be actively suppressed during the larval stage, suggesting that JH may directly or indirectly regulate a component of Hh signaling pathway and silence the expression of Hh target genes, such as dpp and wg.

Expression of dpp and wg, however, appeared to be unaffected by treatment of the body with methoprene for 16 hrs on day four of the final instar. Our results do not rule out the possibility of an interaction between JH and the Hh signaling pathway, because by day four, the tissues may have already committed pupally and were insensitive to JH. In holometabolous
insects, commitment of larval to pupal tissues is spatially and temporally complex and varies
between individuals (Quennedey and Quennedey, 1999). Epidermal tissues, including eyes and
external appendages, are committed before the prepupal ecdysteroid peak (Quennedey and
Quennedey, 1999). Once tissues have committed, they are no longer sensitive to JH (Quennedey
and Quennedey, 1999). Thus, methoprene could be applied earlier or tissue cultures with
methoprene should be conducted in the future to clarify the link between JH and Hh.

**Implications for the origins of metamorphosis.**

The transition from hemimetaboly to holometaboly is poorly understood. By comparing
*Tribolium*, a holometabulous insect, with hemimetabolous insects such as crickets, we are able to
assess the role of Hh signaling in the evolution of metamorphosis. In the cricket *Gryllus*,
knockdown of *hh* has no phenotypic effects on the embryo or the hatched nymph (Miyawaki,
2004). Knockdown of Hh was attempted but no definitive phenotypes were reported although,
the knockdown verification indicated that the animals might be *hh*-RNAi resistant (Miyawaki,
2004). In contrast, during cricket limb regeneration, *hh* does appear to play a role (Mito, 2001;
Nakamura et al., 2008). *hh* expression was found in the limb bud of regenerating cricket nympha
legs, and its removal results in a subtle patterning defects that lead to the duplication of legs
(Nakamura et al., 2008). In contrast, when *hh* dsRNA is injected into *Tribolium* larvae, the
ablated limbs do not regenerate and merely form a blastema-like structure (Villareal, 2013).
Thus, *hh* appears to play distinct roles during normal development and regeneration in *Gryllus*
and *Tribolium*.

The differences in phenotypes seen in *Gryllus* and *Tribolium* suggest that Hh has
divergent functions during regeneration and patterning. Although no phenotypes were observed
during the cricket’s embryonic or nymphal stages, removal of \textit{hh} during the embryonic stage of \textit{Tribolium} results in truncation of limbs (Farzana and Brown, 2008), and we have found that \textit{hh} plays a crucial role in larval limb morphology and the larval-pupal development (this study). Similarly, in \textit{Drosophila}, \textit{hh} plays a key role during imaginal disc development and metamorphosis. Thus, Hh may have gained a novel function during the evolution of holometabolous insects.

We propose that Hh has gained a novel function in creating the morphologically distinct larval appendages. During the larval phase, when JH is high, Hh is inhibited from further elaborating the adult limb morphology. Once JH is cleared from the hemolymph, the limbs are remodeled by the action of Hh. Similarly, over-activation of Hh signaling in the eyes of \textit{Tribolium} larvae led to compound eye development. This would imply that normally, JH represses Hh expression in the eyes in the larvae, and once JH levels decline, Hh is activated, allowing compound eyes to develop.

Our study has implications for our understanding of the evolution of metamorphosis. According to Truman and Riddiford, the larva is an extension of the embryonic stage (Truman and Riddiford, 1999). Truman and Riddiford argued that holometaboly is a result of a heterochronic shift towards the embryonic state (Truman and Riddiford, 1999). A shift in embryonic JH secretion would result in the retention of embryonic features upon hatching (Truman and Riddiford, 1999). However, larval appendages are not simply developmentally truncated embryonic legs. Instead, they have distinct morphologies that are adapted for locomotion and sensing of the environment. In view of the Truman and Riddiford hypothesis, Hh may have gained a novel function in creating the morphologically distinct larval appendages. The change in endocrine expression and acquisition of Hh function together would have resulted
in the evolution of the larval morphology, and in turn, the transition from hemimetabolous to holometabolous metamorphosis. The interplay between Hh and JH expression would have allowed the development of the embryo to the larva, the maintenance of the larval stage, the initiation of the pupal stage, and finally, the adult phase. Thus, according to this hypothesis, the origin of metamorphosis may have its roots in the changes in the expression and functions of JH and the Hh signaling pathway.

In contrast, Hinton suggests that the larva is equivalent to the nymph; likewise, the pupa is akin to the final nymphal stage. In view of this hypothesis, the larval morphology would have evolved through acquisition of Hh patterning during the development of nymphal characters. The acquisition of Hh signaling during nymphal limb development would then have led to the modification of nymphal legs to a distinct larval morphology.

However, neither of these models account for the origin of imaginal cells. We therefore propose that two distinct cell types evolved during the evolution of holometabolism: Hh-dependent cells and Hh-independent cells. We suggest that polymorphic cells evolved through the acquisition of Hh signaling and may have precipitated a dramatic change in the embryonic development. In this case, the larval form may represent an evolutionary novelty that evolved through insertion between the embryonic and adult phases. In hemimetabolous insects, Hh plays little to no role in development (Fig. 10A). An unknown pathway controls development such that tissue commitment and differentiation occurs before hatching (Fig. 10A). Complete differentiation of these embryonic legs results in the formation of nymphal legs that are morphologically similar to the adult legs (Fig. 10A). In holometabolous insects, Hh became co-opted for limb development. These Hh-dependent cells gave rise to the unique larval legs (Fig. 10B). Prior to hatching, JH titers rise, suppressing Hh and further growth of these embryonic
appendages (Fig. 10). Once JH titers decrease, Hh is reactivated, and adult development continues (Fig. 10B). At this point, polymorphic Hh dependent cells and Hh independent cells, which are suspected to be imaginal cells, commit to adult tissues, resulting in complete differentiation (Fig. 10B). Thus, we propose that the co-option of Hh signaling pathway in insect development led to the development of the novel larval form found in holometabolous insects (Fig. 10).

Our present study cannot distinguish between the three models. Regardless of the theories, our study suggests that the acquisition of JH-sensitive Hh signaling in the patterning of imaginal cells may have led to the evolution of the specialized larval morphologies. Future studies on additional hemimetabolous insects, such as Oncopeltus, will provide more definitive support for this hypothesis.
Figure 10. Proposed model for the evolution of holometabolous insects. A) Role of Hh in hemimetabolous insects. B) Role of Hh in holometabolous insects.

**A)** Role of Hh in hemimetabolous insects.

**B)** Role of Hh in holometabolous insects.
Concluding remarks and speculations

In summary, this study examines the role of the Hh signaling pathway in tissue growth, cell proliferation, and overall insect development. Our findings indicate that the expression and suppression of Hh are key in tissue growth and proper axis patterning. Previous studies have shown that Hh is a highly conserved pathway used for regulating cell proliferation and development. In *Drosophila*, Hh signaling is required for leg development, the eye primordium, and the maintenance of the ovarian somatic stem cell population (Kojima, 2004; Royet and Finkelstein, 1997; Zhang and Kalderon, 2001). Similarly, in mammals, Hh has been found to be a regulator of endodermal cells, such as the intestinal epithelium (Ingham, 2011; Ramalho-Santos et al. 2000). Similar to the ectopic eyes seen in our ptc- and cos2- knockdown animals, overexpression of Sonic hedgehog, the mammalian homolog of Hh, can result in craniofacial duplication (Roessler et al, 1996). Thus, aspects of Hh signaling in insects and vertebrates are likely to be evolutionarily conserved.

Although the pathway is highly conserved across the metazoan, Hh functioning as a regulator of larval limb growth may have been a novel acquisition in holometabolous insects. The recruitment of JH-sensitive Hh signaling in polymorphic cell proliferation and development may underlie the evolution of the distinct larval morphology that may have precipitated the spectacular diversification of holometabolous insects we observe today.
REFERENCES


