Unraveling The Genetic Mechanisms Involved In The Evolution And Development Of The Thoracic Appendages In Insects

Victor Medved
Wayne State University,

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UNRAVELING THE GENETIC MECHANISMS INVOLVED IN THE EVOLUTION AND DEVELOPMENT OF THE THORACIC APPENDAGES IN INSECTS

by

VICTOR MEDVED

DISSERTATION

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Approved By

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Advisor Date

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DEDICATION

This work is dedicated to my parents Mijo and Kata, my sister Susan, my brother Mike and my nephews Michael, Nicholas and Ryan for their support throughout my graduate training. I would also like to dedicate this work in loving memory of my grandmother Ana Marković.
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CHAPTER 1
INTRODUCTION

Arthropods represent the most morphologically diverse animal phylum, and have been able to successfully adapt to almost every available ecological niche and environmental condition. Of the four major arthropod groups, the insects display the highest degree of morphological variation, particularly in their appendages. These characteristic appendage traits may range from the size and shape of a whole leg or particular leg segment(s) to the texture and pigmentation of a wing. In regard to legs, the analysis of their morphology in primitive, apterous species shows that highly similar legs are present on each of the three thoracic segments (T1-3). The subsequent divergence observed during insect radiation is the result of modifications to this original state with all legs being uniform. To a large degree, this trend can be observed in cockroaches whose legs are highly similar and adapted for running – while featuring a robust, muscular proximal-most segment (coxa), which is greatly reduced in other insects. In the praying mantis, on the other hand, the T1 leg is morphologically unique and modified for prey capture, while the hind legs are elongated, slender and used for walking. In mole crickets we can observe another specialized T1 leg morphology, which is modified for burrowing. In general, the second pair of thoracic legs in insects are unmodified and are usually thought of as having default leg morphology. The T3 leg displays perhaps the greatest degree of variation in its size and function, ranging from the enlarged jumping legs in crickets and grasshoppers to the specialized pollen
gathering leg in bees. Analogous to legs, wings in the most basal pterygote group (dragonflies) are similar in size and shape and are considered to represent the ancestral wing condition. The subsequent changes in wing morphology were so significant that they were used as the basis for the individual taxon names given to many insect orders. For example, in Hymenoptera (bees and wasps), both the fore- and hindwings are membranous. In Coleoptera (beetles), the forewings are modified to form the elytra, which is a completely hardened structure that serves as protection to the membranous hindwings. Another variation is observed in Hemiptera (true bugs) in which the forewings are only partially hardened (hemelytra), while the hindwings remained membranous. Perhaps the most extreme modification is seen in the Diptera (flies) in which the hindwings have been completely reduced and transformed into balancing organs (halteres). The general theme that emerges from these comparative analyses is that, regardless of the appendage, there is a common wing or leg morphology that is being modified in a lineage-specific fashion.

To a large degree, the above mentioned differences in legs or wings can be explained by alterations in the expression, function and regulation of genes that specify the identity of thoracic segments and their associated appendages (Angelini and Kaufman, 2004, 2005; Angelini et al., 2005; Carroll et al., 1995; Hughes and Kaufman, 2002; Mahfooz et al., 2007; Passalacqua et al., 2010; Rogers et al., 1997; Ronshaugen et al., 2002). In *Drosophila* and *Oncopeltus*, the *Sex combs reduced* (*Scr*) is responsible for the combs that are unique to the T1 leg (Chesebro et al., 2009; Pattatucci and Kaufman, 1991). Expression studies suggest that this gene may also be involved in the evolution of the raptorial T1 leg in mantids (Passalacqua et al., 2010).
Therefore, Scr appears to be acting as a common modifier of the T1 leg in insects. On the other hand, studies in Drosophila have shown that Ultrabithorax (Ubx) controls the identity of the entire T3 segment, including hind legs and hindwings (Lewis, 1978; Pavlopoulos and Akam, 2011; Stern, 2003; Struhl, 1982). Combined with experiments in several other species, subsequent analyses have shown that Ubx mutations result in hindwings and T3 legs assume the identity of forewings and T2 legs, respectively (Khila et al., 2009; Mahfooz et al., 2007; Tomoyasu et al., 2005). Therefore, similar to the previously described roles of Scr, Ubx seems to act to provide a distinct species-specific morphology to T3 dorsal and ventral appendages.

The above results revealed that while hox genes act as global regulators responsible for establishing the identity of a particular leg or wing, it is the changes in expression or function of their downstream targets that likely generate the actual differences observed in these appendages. For example, Scr was shown to differentially regulate the expression of leg patterning genes such are Distal-less (Dll) and dachshund (dac), which in turn, control the number of combs in various Drosophila species (Atallah et al., 2014). In Tribolium, Ubx is responsible for the formation of a unique membranous hindwing by suppressing apterous (ap) and Achete-scute homologue (ASH), genes that are involved in tissue sclerotization and are normally upregulated in forewings (Tomoyasu et al., 2009). At present, there are only a few examples of identified targets for Scr and Ubx in any insect species. The unanswered question is, when considering morphological adaptations observed in a given species’ appendage, how much of a downstream target set is common and how much is lineage-specific?
To begin to address some of the above issues, we first focused on another example of a greatly modified T3 leg, the pollen gathering leg in honeybees. When compared to the forelegs, the hind leg in *Apis* has a greatly enlarged tibia and basitarsus. The tibia is concave and bristle free to allow for the transport of pollen, while the bristles of the basitarsus are arranged in neatly spaced rows and used for grooming and collecting. In light of our previous studies of insect T3 legs (Mahfooz et al., 2007; Mahfooz et al., 2004), we examined if *Ubx* also regulates the changes in morphology seen in the T3 leg. Honeybees are divided into three different castes: drones (males), workers and the queen. The workers and queen, which are both female, have similar morphologies with the main difference being the corbicula (pollen basket) present on the hind legs of the worker. To examine if *Ubx* plays a role in this caste specific difference, we performed RNAi experiments to deplete its expression in the developing *Apis* larvae. Our results show that *Ubx* is indeed responsible for the formation of the corbicula and related structures (pollen press and comb) in workers (Medved et al., 2014). To further determine if the role of *Ubx* in corbicula development is unique to *Apis* we examined its expression in another member of the corbiculate bee clade, *Bombus impatiens* (bumble bee). Significantly, *Bombus Ubx* is also expressed in a similar pattern to *Apis*, suggesting that its involvement in the formation of the pollen basket was a key event that allowed for the social interaction in corbiculate bees.

Insect wings offer a unique opportunity to examine how novel structures originate and further diverge from one another. However, to begin to address these issues it is necessary to first consider the origin of wings. For more than a century, the debate has centered around two competing theories. The first is the paranotal theory, which argues
that wings originated from the lateral outgrowths of the body wall (Rasnitsyn, 1981).

The second theory is the exite theory, which postulates that wings originated from dorsal gill branches (epipods) found on the legs of crustaceans (Averof and Cohen, 1997). Recently a new combinatorial model has been proposed that marries these two original theories (Niwa et al., 2010). This model proposes that wings originate from two separate modules, a dorsal (notal expansion) and ventral (epipodite) component. Experiments in Tribolium provided the first physical evidence for this new model, showing how two separate and distinct elements (one dorsal and one ventral) fuse to create an ectopic T1 wing in Scr RNAi beetles (Clark-Hachtel et al., 2013). This result, though, was obtained from a single species, which belongs to a derived insect group (Coleoptera, beetles) that undergo a modified, holometabolous mode of development.

To determine to what extent these observations are lineage-specific or if they represent a general trend in insects we performed the analogous experiments in a hemipteran, Oncopeltus fasciatus. The hemipterans represent phylogenetically more basal insects and they also undergo an ancestral (hemimetabolous) mode of development. First, we performed RNAi experiments in Oncopeltus, focusing on the same set of genes that were previously used in Tribolium. Our analysis shows that in addition to affecting wing development, some of these genes also cause alterations in the scutellum (the dorsal plate on T2). This finding of a shared gene network between the wings and dorsal plates provides a first insight into the genetic mechanisms that may be involved in co-evolution between these structures. In addition, RNAi of wing genes in Oncopeltus had an effect only on the dorsal portion of the T1 segment. This is quite different from the situation previously reported in Tribolium, in which both the dorsal and ventral portions
of T1 were affected. These differences between *Tribolium* and *Oncopeltus* are significant, because previous studies postulated that the tissues affected in T1 segment represent wing serial homologs (Clark-Hachtel et al., 2013; Ohde et al., 2013). Second, we also performed transcriptome analysis of the developing (T2 and T3) wings and T1 ectopic wings in *Oncopeltus*. Our study provides independent support for the combinatorial model of wing origins, showing that *Oncopeltus* fore- and hindwings are composed of both dorsally and ventrally expressed genes. Furthermore, the transcriptome data reveal that T1 ectopic wings are of strictly dorsal origin, validating our RNAi results. Surprisingly, the gene most highly expressed in T2/T3 wings but completely absent from the ectopic T1 wing was *tracheless* (*trh*). This gene has been identified as an arthropod ventral appendage gene (Franch-Marro et al., 2006) and is a master regulator for tracheal development in insects (Sánchez-Higueras et al., 2014). This may be a significant discovery because in terms of its structural integrity, wing tissue cannot stiffen and expand in size in the absence of trachea (Chung et al., 2011). Therefore, the lack of ventral origin genes (such as *trh*) in the T1 wing may be the reason for its small size and subsequent disappearance.

Overall, the present results in *Oncopeltus* provide a framework than can be extended to other basal, hemimetabolous lineages. Such broadened insight will allow us to directly establish a consensus as to what are the main components of the dorsal wing program, while indirectly also gaining knowledge regarding the ventral wing components. This combined information, in turn, will enable the future functional studies of identified candidate genes and determination of their relative contributions to wing divergence and evolution.
CHAPTER 2

_Ubx_ promotes corbicular development in _Apis mellifera_

The contents of this chapter have been published in _Biology Letters_.


Abstract

The key morphological feature that distinguishes corbiculate bees from other members of the Apidae family is the presence of the corbicula (pollen basket) on the tibial segment of hind legs. Here, we show that in the honeybee (_Apis mellifera_), the depletion of the gene _Ultrabithorax (Ubx)_ by RNAi transforms the corbicula from a smooth, bristle-free concave structure to one covered with bristles. This is accompanied by a reduction of the pollen press, which is located on the basitarsus and used for packing the pollen pellet, as well as a loss of the orderly arrangement of the rows of bristles that form the pollen comb. All these changes make the overall identity of workers’ T3 legs assume that of the queen. Furthermore, in a corbiculate bee of a different genus, _Bombus impatiens_, _Ubx_ expression is also localized in T3 tibia and basitarsus. These observations suggest that the evolution of the pollen gathering apparatus in corbiculate bees may have a shared origin and could be traced to the acquisition of novel functions by _Ubx_, which in _Apis_ were instrumental for subsequent castes and behavioral differentiation.
Introduction

Social insects are widely recognized for their complex behaviors and division of labor within the collective. The best studied social species is the Western honey bee, *Apis mellifera*, which has three castes: the queen, drones, and workers, as is the case for all other *Apis* species. Workers are characterized by having a distinct feature, the corbicula (pollen basket) that they use for packing pollen and transporting it to the beehive. The corbicula is localized on the flattened and enlarged tibia of hind legs and is found in other eusocial bees such as bumblebees and stingless bees. In contrast, this structure is much less elaborate or completely lacking in solitary and less socially complex bee species (Michener, 2000). Therefore, the evolution of the pollen basket has been recognized as a major morphological innovation in social insects (Cardinal and Danforth) and is tied to the development of more complex social behaviors within the Apidae.

Morphological diversification of insect hind legs has been associated with changes in the expression of the hox gene *Ultrabithorax, Ubx* (Bomtorin et al.; Hughes and Kaufman, 2002; Khila et al., 2009; Mahfooz et al., 2004; Struhl, 1982; Tomoyasu et al., 2005). Functional studies subsequently confirmed that *Ubx* directly regulates the size of individual leg segments in a number of insects (Khila et al., 2009; Mahfooz et al., 2007; Stern, 2003). These data, coupled with recent findings showing differential expression of *Ubx* between workers and queens (Bomtorin et al.) led us to test the hypothesis that this gene may play a functional role in the development of the pollen gathering apparatus in honey bees. In addition, we examined *Ubx* expression in the
bumble bee *Bombus impatiens* to infer the generality of corbicular development in Apidae.

**Results**

Honey bee workers transport pollen on the tibial segment of the T3 leg (Fig. 2.1a), which is distinguished by having a concave, naked cuticle region that defines the corbicula (Fig. 2.1b). Except for a single central bristle (white arrow) thought to stabilize the pollen pellet, this area is completely bristle-free. In contrast, the queen lacks a corbicula and has a tibia that is covered in bristles (Fig. 2.1b). The other segment that is involved in pollen gathering is the T3 basitarsus, which is located distal to the tibia. In workers it features 11 neatly spaced rows of bristles that make up the pollen comb as well as a protrusion known as the pollen press (Fig. 2.1b, black arrowhead). Both of these features are absent in queens.

To determine the role of *Ubx* in corbicular development, two non-overlapping *Ubx* fragments were independently injected, yielding similar results; a moderate or strong T3 leg phenotype was observed in 36% of the emerging *Ubx*-RNAi adults (Fig. 2.1b). In moderate phenotypes, the tibial segment lost the central bristle while gaining several ectopic bristles (white arrowheads) in the previously naked cuticular area. In strongly affected individuals the whole corbicular region became completely covered with bristles. Interestingly, the tibia’s overall size and its distinct triangular shape remained unchanged indicating that the function of *Ubx* at this developmental stage is primarily in the suppression of bristle development.
Within the T3 basitarsus (Fig. 2.1b), the distinct rows of bristles that make up the pollen comb were either reduced in number (moderate phenotype) or they completely lost their organization (strong phenotype). In addition, the pollen press was reduced in size (Fig. 1b, black arrowhead). These combined results in the T3 tibia and basitarsus reveal that Ubx plays a critical role in the development of the morphological features that enable pollen collection in Apis.

To determine if this function of Ubx may exist in other corbiculate bees, we examined its expression in the leg discs of Bombus impatiens, which belongs to tribe Bombini (bumble bees). In this species as well, the T3 tibia and basitarsus are modified for pollen collection. As shown in Fig. 2.2a, the Ubx expression is restricted solely to these two segments suggesting that Ubx may play a general role in the formation of the bee’s pollen gathering apparatus.

Discussion

The smooth bristle-free surface of corbicula in Apis (Fig. 2.1b) is reminiscent of the “naked valley” region on the T2 legs in several Drosophila species (Stern, 1998). In the latter instance though, the smooth cuticle is generated by the suppression of the trichomes, which are simple cuticular extensions produced by epidermal cells. However, the honey bee worker pollen basket is generated by inhibiting development of bristles, which are sensory organs (Bomtorin et al.). This suggests that the pollen basket in corbiculate bees results from a novel role of Ubx in the suppression of bristles on the T3 tibia. In honeybees this new role of Ubx appears to be under the control of
**Figure 2.1.** (a) Adult worker bee collecting pollen, the white arrow indicates corbicula filled with pollen granules. (b) Effects of *Ubx* RNAi on adult worker T3 leg morphology. The white arrow points to the central bristle in wild type tibia, while white arrowheads point to ectopic bristles in the moderate phenotype. The pollen press on T3 basitarsus is indicated by a black arrowhead in worker bees, while the asterisk denotes the absence of this structure in queens.
environmental factors (ie. diet) which leads to varying hormonal levels, resulting in phenotypic plasticity giving rise to the different castes.

Two recent studies (Barchuk et al., 2007; Bomtorin et al.) examined the global changes in expression between *Apis* queens and workers and identified a number of genes that are differentially expressed between the castes. While the functional data are still lacking, some genes such as *grunge* (*gug*) and *Ataxin-2* (*Atx2*) play a role in the formation of bristles in *Drosophila* (Al-Ramahi et al., 2007; Erkner et al., 2002). Since *Ubx* has been shown to regulate the development of specific bristles in the fly thorax (Rozowski and Akam, 2002), *gug* and *Atx2* represent prime candidates as possible *Ubx* targets. Another avenue to explore would be to characterize the role of *Ubx* in generating distinct T3 basitarsus phenotypes among the castes. As shown in Fig. 1b, while the basitarsus in workers is organized in 11 distinct rows of bristles, in *Ubx*-RNAi adults this pattern is completely disrupted and resembles the situation present in queens. The observed differences in RNAi phenotypes between the T3 tibia and basitarsus suggest the presence of two modes of regulation by *Ubx*. In the former, *Ubx* may have a direct role in bristle suppression (as it is expressed in workers but not in queens). In the latter, the role of *Ubx* in the spatial organization of bristles may be at the downstream target level instead (since its expression in the basitarsus is common to both castes).

As a way of furthering our understanding of the evolution of the corbicula, we propose a two-step process outlined in Figure 2.2b. The first step involves the divergence of the T3 tibia from its foreleg counterparts and the establishment of its distinct morphology. This is accomplished by the enlargement of the segment in its
Figure 2.2. (a). UbdA expression in *Bombus impatiens* (bumblebee) leg discs showing expression in the tibia (Ti) and basitarsus (Ta-1) of T3 legs. (b) Proposed two-step model for the development of the corbicula.
distal half and the acquisition of a triangular shape. A similar differential enlargement is also seen in the hind legs of non-social bees, suggesting that the T3 enlargement preceded the formation of the corbicula (Michener, 2000). The second step involves the actual creation of the pollen basket, whose presence is a distinguishing trait between the castes (such as workers and queens). As shown in Fig. 2.2b, this step is directly regulated by Ubx through its repression of bristle development generating a naked cuticular region. The finding that Ubx expression in Bombus is also restricted to the T3 tibia and basitarsus raises the possibility that this gene may act as a common trigger in the development of the pollen gathering apparatus in corbiculate bees. Further comparative studies in non-corbiculate bees will be necessary to confirm this hypothesis and in turn, will provide insight of how the acquisition of novel functions by Ubx may have facilitated the evolution of the social behavior.

Material and methods

RNA Interference

Double stranded RNA was prepared via a previously established protocol as described in Mahfooz et al. (2007). In short, two non-overlapping Ubx fragments directed to the 5’ and 3’ ends of the gene were injected independently to control for non-specific or off target effects. The 309 bp long 5’ fragment was amplified with forward 5’ ACTCGTATTTTTGAGCAGACTGCG and reverse 5’ GTCGAATACGAGGATGTCGTG primers. For the 3’ fragment the following primers were used: forward 5’ ACCATACGTTCTACCCCTGGATG and reverse 5’ AGCAAGTCGAGGAACCTAGCG,
Figure 2.3. RT-PCR analysis of *Apis Ubx* mRNA. Lane 1 is wild-type *Apis* larvae, which shows high levels of *Ubx*. Lane 2 is larvae 3 days post injection, which shows a greatly reduced level of expression in *Ubx*-RNAi adult.
generating a 265 bp product. Ten clones of each fragment were recovered, sequenced and compared with each other as well as the existing *Apis Ubx* gene (GenBank accession number XM623986). The injections were performed on L2 and L3 stages of worker larvae with a Picospritzer II (Parker). Approximately 2 µl of *Ubx* dsRNA (5µg/µl) was injected (duration of 60 msec and nitrogen pressure at 20 psi) in five independent trials. We verified the extent of *Ubx* depletion by RT-PCR analysis (Figure 2.3). After injection, the larvae were maintained and reared via an established protocol (Huang da et al., 2009). Overall, a total of 270 L2-L3 larvae were injected, 38 (14%) of which survived to adulthood. Among the surviving adults, 14 (36%) displayed either a strong or moderate phenotype. These survival rates are in line with similar *Ubx* studies in *Tribolium* in which larval injections caused lethality at the pupal stage, while embryonic injections had 20% survival (Tomoyasu et al., 2005). In addition, mortality rates of 50% or more have been reported for RNAi experiments in *Apis* (Kucharski et al., 2008; Wolschin et al., 2011), irrespective of the method used (injections or feeding). To further address non-specific effects we injected a previously cloned 710 bp fragment of the jellyfish Green Fluorescent Protein, GFP (Hrycaj et al., 2010), and followed the previously described control protocols in *Apis* (Page et al., 2006). A total of 50 L2-L3 stage larvae were injected, of which 15 individuals (30%) survived; all resulting adults showed wild-type phenotype.

**Antibody staining**

FP6.87 mouse antibody was used to detect the *Ubx* staining in *Bombus* leg discs, as described in Mahfooz et al (2004).
CHAPTER 3

Genetic mechanisms underlying the origin and diversification of insect wings and thorax

The contents of this Chapter is being prepared for submission to a peer-reviewed journal.

Victor Medved, James H. Marden, Howard W. Fescemyer, Joshua Der, Najmus Mahfooz, and Aleksandar Popadić 2015: Genetic mechanisms underlying the origin and diversification of insect wings and thoracic plates.

Abstract

A robust understanding of insect wing evolution must encompass wing origins, disappearance of wings from the first thoracic segment (T1), divergence of wing structure and function on the second and third thoracic segments (T2, T3), and acquisition of a precise fit between wing edges and adjacent thoracic plates in neopteran insects that fold their wings at rest. Here, we combine functional testing of key homeotic and wing specification genes with RNAseq analyses of developing wings in the hemipteran insect Oncopeltus fasciatus. We show that segment-specific differences in a common wing program account for major trends that shaped the evolution of dorsal appendages in modern insects. First, the small ectopic wing serial homologs on T1 of Scr-i RNAi insects match T2 and T3 wings in expression of many wing-specific genes, but lack genes of ventral origin (e.g. nubbin, tracheless, ventral veinless) that are otherwise upregulated in T2 and T3 wings compared to body wall. In the T1 ectopic wing, 50% of the most highly wing-biased genes are not expressed, thus
creating in essence half a wing reminiscent of T1 structures found in hemipteroid and other early fossils; this may explain why T1 wings were invariably small and disappeared independently in multiple insect lineages. Second, known wing selector genes vestigial, scalloped and nubbin not only control wing development, but also regulate formation of the scutellum, a centrally positioned triangular structure on the dorsal mesothorax (T2). A tight fit between the folded wing edges and scutellum may have been a key evolutionary specialization for terrestrial life after the origin of neopteran insect lineages. Third, the hox gene Ubx regulates distinct changes in shape, size, and coloration of hind wings and also suppresses the development of scutellum on the metathorax (T3). Hence, these results support an emerging model of wing origins in which ventral appendage-associated cells migrated to interact with dorsal cells to make fully formed wings. Divergence of fore and hindwings can be explained in terms of a common T2 wing program that establishes lineage-specific forewing morphology, while Ubx acts as a general hind wing selector gene responsible for the establishment of lineage-specific hind wings.

Introduction

The most extensive evolutionary radiation in animals followed the origin and diversification of insect wings. Whereas modern insects have fore-(T2) and hindwings (T3), a number of early fossil species had wings on all three thoracic segments (Wootton and Kukalova-Peck, 2000), including the prothorax (T1). After the radiation of pterygote insect orders, the development of T1 wings was repressed in multiple lineages, leading to the present day condition of two wing pairs. Following these
events, fore and hind wings diverged in shape and function (Fig 3.1). Complementary to diversification of wing morphology were changes in the way wings are folded at rest, as evidenced during the evolutionary transition from paleoptera (mayflies and dragonflies), which rest with wings extended from the body, to neoptera, which rest with wings folded and held flat against the body. A precise mechanical fit between the resting wing and body required modifications of both forewing shape and elements of dorsal thoracic plates. Mechanisms governing such changes have yet to be identified. Hence, a robust understanding of insect wing evolution must encompass wing origins, T1 wing disappearance, T2/T3 wing divergence, and coordinated evolution of a precise fit between wing edges and adjacent thoracic plates.

Previous developmental studies revealed mechanisms that underlie wing presence/absence on different body segments and contributed to hypotheses about wing origins (Carroll et al., 1995; Lewis, 1978; Struhl, 1982; Tomoyasu et al., 2005). More recently, the ability to restart the wing program on T1 provided novel insights regarding modifications of the dorsal thorax (notum) in early evolution of wings (Chesebro et al., 2009; Clark-Hachtel et al., 2013; Hrycaj et al., 2010).

In two beetle species, the wing “master gene” vestigial (vg), as well as two other key wing genes scalloped (sd) and nubbin (nub), were shown to have specific effects on both dorsolateral and ventral structures of T1 (Clark-Hachtel et al., 2013; Ohde et al., 2013). These observations support the hypothesis that vg-dependent tissues might represent wing serial homologs in T1, consistent with developmental patterns in mayflies (Niwa et al., 2010). Together, those studies stimulated a new theory of the origin of insect wings. Rather than strengthening or refuting either of the two long-
competing hypotheses that pose either a dorsal origin (paranotal lobe hypothesis; (Grimaldi D, 2005; Rasnitsyn, 1981)) or a ventral appendage origin (wings-from-gills; (Averof and Cohen, 1997; Kukalova-Peck, 1978)), these new results indicate that wings arose from a fusion of dorsal and ventral components of thoracic plates. However, functional data supporting such a combinatorial model has been obtained from only a single holometabolous order (Coleoptera, beetles). The highly derived nature of holometabolous development, featuring a pupal stage during which larval tissues are broken down and very different adult tissues are formed, highlights the significance of analyzing more basal, hemimetabolous species (direct development without a pupal stage) in order to determine to what extent these observations are lineage-specific or represent a general trend in insects.

To investigate further the genetic mechanisms governing the morphological evolution of dorsal thorax and its associated structures, and their subsequent functional integration in modern insects, we used a direct-developing hemipteran species, the milkweed bug (*Oncopeltus fasciatus*; Hemimetabola, Hemiptera). We first utilized a candidate gene approach in which we depleted (via RNAi) the expression of the *Oncopeltus* orthologs of wing specification genes *vg*, *sd*, and *nub*. Second, we used a nonbiased, global approach (utilizing RNAseq analysis (Wang et al., 2009) to characterize all expressed genes in wild type T2 and T3 wings, ectopic T1 wings, and wild type T1 body wall in order to provide independent and expanded insights regarding wing origins and their subsequent divergence.
**Figure 3.1.** Major events in the divergence and diversification of insect wings. Note the presence of a T1 winglet in fossil insects, which persisted until after T2 and T3 wings elongated. Experimental inhibition of homeotic genes generates an ectopic T1 winglet (Scr RNAi) and a return to identical T2 and T3 wings (Ubx RNAi), thereby recapitulating ancestral states driving key evolutionary transitions.
Results

Function of wing specification genes in the prothorax

The wild type dorsal pronotum (T1) in *Oncopeltus* is trapezoidal in shape, characterized by a wider posterior half (Fig. 3.2A). In adult insects from nymphs treated with RNAi to inhibit key wing genes (*vg, sd* and *nub*), the main effect was localized in this region with *vg* RNAi displaying the strongest phenotype (~10% width reduction). As shown in Fig. 3.2B-D (and associated overlays, 3.2B’-D’), the affected area encompassed the orange-yellow colored dorso-lateral edges, just dorsal to the junction with the ventro-lateral sternum. In contrast, no large effects were observed on the ventral T1 plates, including the sternum and epimeron (Fig. 3.2E-H; E’-H’). The only change on the ventral prothorax was restricted to an area surrounding the leg base, comprising incomplete fusion of the pleural suture and a notch that increased in size in the *nub*-RNAi adults (compare Fig. 3.3B vs. 3.3E). Hence, the functions of wing orthologs in *Oncopeltus* T1 are mainly localized to the dorsal pronotum, with additional subtle effects of *nub* on ventral leg-adjacent structures of the prothorax.

Effect of wing genes on T2 and T3

Adult T2 morphology is comprised of semi-membranous wings (hemelytra) that fit tightly against a large triangularly shaped scutellum on the dorsal side (Fig. 3.4A-B and 3.4F, J). Tucked beneath the hemelytra is a pair of membranous T3 hindwings, featuring a distinct shape and pigmentation (Fig. 3.4B). Both pairs of wings in *vg*-RNAi adults exhibited significant alterations in their shape and size, especially the hindwings, which became greatly reduced (Fig. 3.4C). The *sd* RNAi wings displayed similar
changes, but to a lesser degree (Fig. 3.4D), consistent with studies in *Drosophila* where the wings of *sd* mutants do not result in a complete loss of wings (Campbell et al., 1992). The depletion of *nub* also caused a great reduction in the fore- and hindwing size, and a different forewing shape (Fig. 3.4E).

In addition to the observed effects on wings, these genes also affected the scutellum, the centrally positioned triangular structure on the dorsal mesothorax. No such effects were previously observed in beetles (Clark-Hachtel et al., 2013; Ohde et al., 2013), possibly because radical reconfiguration of the thorax during the pupal state in holometabolous insects may override any such effects. In *Oncopeltus vg* RNAi adults, the scutellum lost its tip and changed its distinct triangular morphology into a shield-like shape (Fig. 3.4G). *sd* RNAi also affected the posterior half of the scutellum, but to a lesser degree; these adults displayed a less pronounced shield-shape (Fig. 3.4H). In *nub*-RNAi adults, the result was somewhat different: in this instance, the scutellum was not affected but there was a curving of the forewing clavus, preventing the wings from laying flat (Fig. 3.4M). In all cases, the altered morphology of the scutellum and wings disrupted the snug fit between these structures and prevented the forewings from laying flat on the body (Fig. 3.4K-M). These results are the first indication that the master genes controlling wing development also affect the shape of adjacent thoracic structures that interact functionally with wings to achieve a precise mechanical fit during rest.

To further investigate the control of notal tissue adjacent to the wings, we dissected the T2 wing pad of 5th instar nymphs, which has the shape of a horseshoe (Fig. 3.4A', inset; Fig. 3.5) and constitutes one contiguous structure. To determine
Figure 3.2. Effects of vg, sd and nub RNAi on the prothorax (T1) of Oncopeltus fasciatus. (A-D) The fronto-dorsal view of: wild-type (A), vg RNAi (B), sd RNAi (C), and nub RNAi (D). (A’) The outline of the wild-type pronotum. (B’-D’) The overlay of vg RNAi, sd RNAi and nub RNAi, respectively (grey) onto wild-type (black). (E-H) The ventral view of T1 sternum in: wild-type (E), vg RNAi (F), sd RNAi (G), and nub RNAi (H). (E’) The outline of wild-type sternum. (F’-H’) The overlay of vg RNAi, sd RNAi and nub RNAi, respectively (grey) onto wild-type (black).
**Figure 3.3.** A. Fronto-ventral view of T1 ventral plate. B-E. Close-up of region outlined in A in a wild-type, vg RNAi, sd RNAi and nub RNAi, respectively. F. Lateral view of T1 plate. G-J Magnified detail of the outlined region in wild-type G, vg RNAi H, sd RNAi I and nub RNAi J, respectively. The asterisk in J shows a notch created in the nub RNAi treatment.
**Figure 3.4.** The functions of wing genes in the mesothorax (T2) of *Onopeltus*. (A) Dorsal morphology of the adult T2 segment, arrowheads point to the clavus of the forewings. (A’) The dissected dorsal T2 plate of a fifth nymph. The dotted line denotes the proximal-most boundary of wing primordia. (B) Wild-type fore- and hindwings. (C-E) The effects of *vg*-RNAi, *sd*-RNAi, and *nub*-RNAi on adult wing morphology, respectively. (F) Dissected dorsal T2 notum of wild-type adult. (G-I) The effect of *vg*-RNAi, *sd*-RNAi, and *nub*-RNAi, respectively. (J-M) The alignment of the scutellum and clavus of the forewing in wild-type (J), *vg*-RNAi (K), *sd*-RNAi (L), and *nub*-RNAi (M) adults, respectively. In panels K-M, this alignment is disturbed causing scutellum and clavus to lose their close contact to one another. The created open space is artificially colored in green.
which regions of the wing pad give rise to thoracic structures, we performed heat lesions on its central region (Fig. 3.5). In the resulting adults, wings were not affected whereas the scutellum had a misshapen morphology – showing that the central region gives rise to the scutellum. We also performed RT-PCR analysis on the T2 wing pad and found \textit{vg} expression in both the central and lateral regions (Fig. 3.6). In contrast, \textit{nub} expression was observed only in the latter. These observations are consistent with the above RNAi phenotypes, where \textit{vg} affects both the wings and scutellum while \textit{nub} affects only forewings.

The hemipteran wing and scutellum apparently develop from a common primordium in which \textit{vg} straddles the proximal-distal axis, regulating both the scutellum (proximal portion) and wing (distal portion), whereas \textit{nub} is expressed only in the distal portion that becomes the wing blade. In this regard, it is interesting to note that expression of \textit{nub} is similarly restricted to the posterior compartment of crustacean gills (Franch-Marro et al., 2006), which our results suggest may be homologous to the insect wing blade.

\textbf{Ubx function in wing divergence}

In regard to thorax segmental identity, T2 is believed to be the ground state and changes to the default T2 morphology were required for divergence of the thoracic segments and their appendages (Lewis, 1978). In \textit{Drosophila}, loss of Ultrabithorax (\textit{Ubx}) results in the T3 segment assuming a T2 identity, including the striking transformation of halteres (balancing organs) to fully developed forewings (Bender et al., 1983; Pavlopoulos and Akam, 2011). Similarly, \textit{Ubx} inhibition in \textit{Tribolium} transforms the membranous hindwings to forewing-like sclerotized elytra (Tomoyasu et
Figure 3.5. (A) Dorsal view of a T2 wing pad in wild type 5th nymph. (B) Final adult T2 morphology with the focus on scutellum (scu). (C) The heat lesions administered to the central region of 5th nymphs (green highlighted region in panel A) result in malformation of the scutellum in adults (D-D3).
**Figure 3.6.** (Left) The dissected wild type wing pad in 5th nymph, showing a central region (CR) and wing primordia (wg). (Right) RT-PCR showing expression of vg in the CR and wg regions, while *nub* is only expressed in the wg region.
al., 2005). Those results identify *Ubx* as the homeotic gene controlling T3 segmental identity.

In *Oncopeltus*, the membranous, blackish hindwings have diverged evolutionarily from the orange-black hemelytra forewings (Fig. 3.7A-B). The whole metathorax (T3) is also smaller, with the dorsal notum being rather narrow compared to the robust scutellum on T2 (Fig. 3.7C-E). On the ventral side, the meso- and metasternum can also be easily distinguished: the V-shaped posterior half in the former differs from the distinct oval-shaped morphology in the latter (Fig. 3.7F). Consistent with previous results in holometabola, *Oncopeltus* *Ubx*-RNAi adults have T3 wings possessing all key features of T2 morphology, including their distinct shape, texture, and pigmentation (Fig. 3.7A’-B’). This includes the transformation of the proximal anal lobe present in the wild type T3 wing to a clavus-like T2 morphology (blue arrowheads in Fig. 3.7B and B’). In addition to wings, the T3 dorsal notum also showed significant alterations and developed an ectopic scutellum (Figs. 3.7C-D and C’-D’). The extent of the change is best visible in dissected plates of wild type and *Ubx*-RNAi adults (Fig. 3.7E-E’), enabling a full view of their structure. On the ventral side, the oval-shaped T3 sternum (Fig. 3.7F) assumed the V-shaped morphology of the T2 sternum (Fig. 3.7F’). These results show that in a hemimetabolous species, *Ubx* also specifies a unique T3 segment morphology by altering the default T2 segment program. Unlike holometabola, these effects occurred not only on the wings but also the dorsal and ventral plates as well. In terms of its dorsal function, *Ubx* “makes” hindwings by altering the default forewing program while also repressing the formation of a T2 scutellum.
Figure 3.7. The role of *Ubx* in *Oncopeltus* metathorax. (A, A’) Dorsal views of the wild type and *Ubx*-RNAi adults. (B) Wild type fore- and hindwing display distinct morphologies with regards to their shape, color, and size. (B’) While forewings look the same, hindwings are transformed into forewings in *Ubx*-RNAi adults. (C) A side view of the dorsal plates in the wild type showing that the T2 segment features a well developed scutellum. (C’) A side view of *Ubx*-RNAi adult indicates the presence of an ectopic scutellum on T3 (black arrow). (D) Close-up view focused on wild type scutellum. (D’) In *Ubx*-RNAi adults, a second ectopic scutellum forms beneath the T2 scutellum (black arrow). (E) Dissected T3 plate of wild type adult. (E’) Dissected T3 plate of a *Ubx*-RNAi adult. (F) A close-up view of T2 and T3 ventral plates in wild type. The ventral T2 has a triangular shape, while the T3 is oval. (F’) In *Ubx*-RNAi adults, the ventral T3 is transformed into a ventral T2 sternum. Abbreviations: Scu- scutellum; T2- second thoracic segment; T3- third thoracic segment.
Figure 3.7
Discussion

Nature of T1 wings

Previous work in beetles has revealed that the prothorax (T1) contains vestigial (vg) dependent tissue, leading to the proposal that the affected T1 regions represent wing serial homologs (Clark-Hachtel et al., 2013; Ohde et al., 2013). Our present results show that in addition to vg, several other orthologs of genes involved in wing development also have a function in the wingless prothorax of Oncopeltus. By showing that Oncopeltus T1 EW is a unique type of wing and a characteristically T1 structure (Figs. 3.8-3.9 and Table 3.1) (Medved et al., 2015), rather than a conversion to T2 segmental identity, these results establish for the first time the presence of a T1 wing serial homolog in a hemimetabolous insect. In contrast to the situation observed in Tribolium, where T1 EW is derived from both dorsal and ventral tissues and may represent conversion of T1 to T2 segmental identity, wings in Oncopeltus are of strictly dorsal morphological origin and have gene expression profiles indicating absence of ventral-origin cells. (Fig. 3.10) (Medved et al., 2015). Given that the Oncopeltus T1 EW does not express 50% of the most strongly wing-biased genes, it is literally half a wing.

What developmental mechanisms create a fully formed and functional wing?

The recent discovery of the vg-dependent tissue on the wingless prothorax in Tribolium represents a surprising and influential finding in regard to wing origins (Clark-Hachtel et al., 2013). The responding tissue was localized both dorsally (corresponding to the original notal contribution) and ventrally (the original pleural, leg base), suggesting that the wing developmental program may have been at least partially preserved despite the hundreds of millions of years since the wing program was turned
**Figure 3.8.** Gene expression (log₂ [normalized read count + 0.01]) in the three wing libraries compared to the same gene in T1 body wall. Genes undetectable or nearly so in T1 body wall but upregulated > 4-fold in T2 wings are colored pink. Genes down-regulated > 4-fold in T2 wings are shown in green. Arrows identify three individual genes discussed in the text (Medved et al., 2015).
Figure 3.9. Expression in T1 Scr⁻ ectopic wing (A) and T3 (B) wings compared to T2 wings for 109 wing specific genes (undetectable or nearly so in T1 body wall and at least 4-fold higher than minimal expression in T2 wing). Solid line is the line of identity (slope = 1, intercept = 0) and the flat dashed line (A) shows genes (open symbols) missing from the T1 ectopic wing. Note that these same genes, with one exception, are expressed in T3 wing at levels indistinguishable from T2 wing. One gene very highly expressed in all wing types (Moesin) is excluded from this plot because its inclusion necessitates rescaling the axes and reducing clarity of the key result (Medved et al., 2015).
Table 3.1. T2 wing-specific genes not expressed in T1 Scr- ectopic wing (points along the flat line in Fig 3.9a). A value of -6.64 represents undetectable expression (see Fig 3.8 to relate these expression levels to genes overall) (Medved et al., 2015).

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Figure 3.10. Least square means (+ SE) of normalized read counts of (A) a set of wing specification genes (vg, sd, wg, ap) equally expressed in all wing types, and (B) the ventral origin genes nub and tracheal genes (trh, vvl, sal, stat92E) that are missing in the T1 Scr' ectopic wing. These means are from a model that included “gene” and “tissue” as independent variables (Medved et al., 2015).
off in T1 by Scr. To get a more thorough insight into the evolution of wings on T1, it is critical to evaluate these findings in the context of the default wing program on the mesothorax (T2). However, the only T2 effect in Tribolium was observed dorsally, and only in the forewing itself. There were no additional effects on the notal plate, nor any ventral effects. Furthermore, an earlier study in another beetle species (Ohde et al., 2013), Tenebrio molitor (mealworm), also showed no vg function other than in the wing. As suggested by Clark-Hachtel et al. (2013), such lack of additional functions in T2 may be due to the fact that vg-dependent tissue in the notum had already been incorporated into the forewing or it may be missing altogether. Alternatively, the large rearrangement of tissues that occurs during holometabolous development in beetles may be masking additional effects in the adult mesothorax.

Our results in Oncopeltus show for the first time that vg, as well as other genes involved in wing development (such as sd), have a function in the T2 notum, namely its central region - the scutellum. Using the inference from the previous studies sensu stricto (Clark-Hachtel et al., 2013; Ohde et al., 2013), this observation indicates that the scutellum too may be considered a wing serial homologue. Part of the significance of this discovery is that a functional wing requires specialization of adjacent structures, likely including the hinge, muscle attachment points and, in the case of Neoptera, shape changes to accommodate folded wings.

Without broader taxonomic sampling of basal insects, we cannot differentiate whether wing morphogenesis originally involved the scutellum or whether vg alone was co-opted to this structure during neopteran evolution. Under the former scenario, the observed absence of nub in the scutellum primordia may be an ancestral state given its
spatially restricted expression in crustacean ventral appendages (Franch-Marro et al., 2006), a secondary loss of function, or perhaps nub was never recruited to this structure. Distinguishing between these alternatives will require detailed analyses of wing gene expression and developmental roles in a broader sample of paleopterous and hemimetabolous lineages. In any case, gene expression differences between the scutellum and wing blade may comprise key parts of wing folding mechanics that became highly specialized following the origin in neoptera of the ability to fold the wings flat against the body.

**Evolving a lineage-specific T2 wing program and divergence of hindwing**

It has long been held that the T2 wing is the default state and the divergence of wings is the result of modifications to this ground state. This is the case in holometabolous insects (Carroll et al., 1995; Struhl, 1982; Tomoyasu et al., 2005) and here generalized to hemimetabolous insects where the loss of Ubx results in the transformation of hindwings into forewings. However, it is intriguing that the default state of the T2 wing in each case is species-specific. That is, in Drosophila the default state is a membranous wing whereas in Tribolium and Oncopeltus the T2 is an elytra or hemelytra, respectively. While the default state of the wing may vary, Ubx functions the same way in each case by always modifying an existing T2 to create a divergent hind wing (halters in Drosophila or membranous wings in both Tribolium and Oncopeltus). Therefore, the function of Ubx in the divergence of wings is to modify the existing T2 default wing and this role most likely pre-dates any T2 lineage-specific changes in the wing program.
Similar to the way *Ubx* specifies T3 segmental identity, *Scr* controls development of the T1 segment. Small T1 wings are present in a phylogenetically diverse set of fossil insects, but no insects with T1 wings persist in fossils more recent than about 250 mya (Grimaldi D, 2005). Our results in *Oncopeltus*, combined with recent developmental insights from *Drosophila* (Sánchez-Higueras et al., 2014) provide a reason why T1 wings were likely predestined for small size, limited functionality, and independently lost in multiple insect orders. Ventrally originating cells in *Drosophila* embryos migrate dorsally and undergo an epithelial-mesenchymal transition (EMT) to form the corpora cardiaca and prothoracic glands in the head and T1 segments. Serially homologous cells in segments T2 through A8 also migrate dorsally and undergo EMT to form the main tracheal trunks, which extend and branch to serve all parts of the body for gas exchange. It is likely that migration of most of the T1 compliment of these cells to glandular tissue or associated tracheae pre-empted their contribution to T1 wings. Also restricted to the *trh*-expressing ventral-origin cells in *Drosophila* are genes with wing-relevant functions such as muscle attachment (*sls*) and mechanosensing (*f*). Stunting of the T1 wing may therefore demonstrate the limited potential of notal, primarily cuticular cells, to form a large and useful wing without the functions contributed by ventral-origin mesenchyme. In other words, without such a fusion, T1 EW-like wings did not acquire nerves, muscles, or an oxygen supply capable of supporting flapping flight. The gene *ventral veinless* (*vvl*) acts downstream of *trachealless*, suggesting that the ventral component of wing vein formation (critical for wing stiffening) also derives from ventral cells that were diverted to gland formation in the T1 segment, possibly explaining the frequent puckered appearance of T1 wings in fossils.
Tracheal placodes and leg primordia arise from a common pool of cells in *Drosophila* (Franch-Marro et al., 2006), with differences in their fate controlled by the activation state of the wingless signaling pathway. Tracheal cells and spiracles on T2 and T3 arise from locations lateral to the leg bases (Franch-Marro et al., 2006), reminiscent of the association between gills and appendages in crustaceans and strengthened by the finding that homologues of tracheal inducer genes (*trh, vvl*) are specifically expressed in the gills of crustaceans (Chavez et al., 1999; Mitchell and Crews, 2002). Furthermore, *nub* affected leg-adjacent morphology of the T1 ventral *Oncopeltus* thorax and is expressed only in the posterior compartment of crustacean gills (Franch-Marro et al., 2006), consistent with our finding that *nub* is expressed in the blade portion of the *Oncopeltus* wing but not the more anterior portion that regulates scutellum morphogenesis. Hence, starting with fossil evidence (Kukalova-Peck, 1978) and continuing through developmental data (Averof and Cohen, 1997; Clark-Hachtel et al., 2013; Franch-Marro et al., 2006; Niwa et al., 2010; Sánchez-Higueras et al., 2014) and insights here from *Oncopeltus*, the developmental program and osmoregulatory-respiratory function of appendage-associated crustacean gills appears to have evolved into both respiratory (trachea) and locomotory structures in insects when ventral cell populations migrated dorsally to form tracheae and interact with notal cells to form hinged, flappable, stiffened, and elongated appendages (Staniczek et al., 2011) that ultimately enabled flight. After many decades of debate between proponents of the paranotal lobe vs. wings-from-gills hypotheses for insect wing origins, the answer is becoming increasingly apparent; each side was half correct.
In summary, fully sized and functional wings require a fusion of dorsal- and ventral-origin cells, which apparently brought appendage-associated features together with developmental programs that form both the wing blade and coordinately formed dorsolateral body wall. In the first thoracic segment, absence of these ventral-origin cells (possibly diverted to the prothoracic gland) may have doomed T1 wings to partial formation and ultimate disappearance under control of the homeotic gene Scr. Wings on T3 diverged in morphology and function from T2 wings under the control of Ubx. Similar studies in other basal insects, particularly Paleoptera (dragonflies and mayflies), will be required to extend and strengthen this body of evidence and conclusions.

**Material and methods**

**Cloning and sequence analysis of vestigial (vg), scalloped (sd), and nubbin (nub)**

Mixed stage *Oncopeltus fasciatus* (Of) embryos were used for total RNA extraction. The production of cDNA, RT-PCR and cloning were performed according to (Li and Popadic, 2004). To amplify the Of vg fragment, nested PCR was performed using the following primers: outer primers FW 5’GAGGACAGCGGTACTGAAGC, REV 5’TAGTAGGACGCAGGGTCGAG and inner FW 5’ACACTGACGAGGACGAGGAC, REV 5’TACTGCATATTCGCCTGGTG, with the final set of primers yielding a 377 bp fragment. To amplify Of sd, known sequences of sd were aligned and the following degenerate primers were designed for nested PCR: outer FW 5’GAYGARGGIAARATGTAYGGIMG, REV 5’IGTICGDAYIGCICKICCYTCC and inner ATHTAYCCICCITGYGGIMGIMG and REV 5’IACYTTYTCIACIACAYAGYTTICC, with
the final primer set resulting in a 531 bp fragment. The PCR conditions for both vg and sd was 94°C for 2 min; 35 cycles of 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 30 seconds; one cycle of 72°C for 7 minutes. Ten clones from each fragment were isolated and sequenced. The resulting sequences were compared to each other and known sequences from GenBank and were identified as orthologous genes. For Of nub, a previously described 246 bp fragment from (Hrycaj et al., 2008) was used.

RNA interference (RNAi)

Double stranded RNA (dsRNA) was prepared and injected into the abdomens of Oncopeltus nymphs, as described by Chesebro et al. (2009). Approximately 2 µl of dsRNA was injected at a concentration of 2-3 µg/µl into each individual. For vg RNAi a total of 45 individuals were injected of which 35 survived to adulthood. For sd RNAi a total of 75 individuals were injected of which 22 survived to adulthood. For nub RNAi a total of 25 individuals were injected a total of 19 survived. Nymphal RNAi for each gene was carried out consecutively at the fourth and fifth nymphal stages. To address non-specific effects we injected a previously cloned 710 bp fragment of the jellyfish Green Fluorescent Protein, GFP, as described by Hrycaj et al. (2010). To determine the efficiency of our RNAi approach, we performed independent RT-PCR analyses on vg, sd, and nub controls (shown in Fig. 3.11). Oncopeltus 5th nymphs were injected with 2 µl of dsRNA of vg, sd, and nub dsRNA at 2.5 µg/ul and allowed to develop for 3-4 days. Next, the wing pads of two individuals were used for total RNA extraction and cDNA generation, as described by Chesebro et al. (2009). This same procedure was carried out using two wild type individuals. Equal amounts of cDNA from wild type and RNAi 5th nymphs were used as templates in individual PCR reactions to assess the amount
Figure 3.11. RT-PCR analysis of *vg*, *sd* and *nub* mRNA expression levels. Lane 1 shows high levels of * vg* (A), * sd* (B) and * nub* (C) in wild-type *Oncopeltus* 5th nymphs while lane 2 shows greatly reduced levels of expression in the corresponding RNAi nymphs. 18S mRNA levels were used for controls for all three genes.
of relative transcripts that were abolished in injected individuals. As positive controls, primers designed to the *Oncopeltus* 18S ribosomal subunit were used in all cases (Hrycaj et al., 2010).
Chapter 4
Shaping of the T1 Dorsal Notum

Introduction

The prevailing theory of wing origins is the recently established combinatorial model that merges the two previously competing paranotal (dorsal origins) and exite (ventral origins) theories (Clark-Hachtel et al., 2013; Niwa et al., 2010). In Chapter 3, we show that *Oncopeltus* fore- (FW) and hindwing (HW) are composed of cells from both a dorsal and ventral origins, providing for the first time evidence for the combinatorial model in hemimetabolous insects. In addition, our *vg*- and *nub*-RNAi results show that the wing program is still active in the dorsal prothorax, further suggesting that the affected T1 regions are wing serial homologs. Perhaps the most surprising finding involves the function of *vg* in the development of dorsal appendages and structures on T2 segment, revealing that this wing selector gene can act both in wings (FW) and in the neighboring dorsal plate (scutellum). This is significant because while wing development is traditionally considered to be independent from T2 notal development (Cavodeassi et al., 2002; Villa-Cuesta and Modolell, 2005; Zecca and Struhl, 2002), the *vg* RNAi results suggest that these processes actually may be related. To further explore this idea, here we focus on the transformations of the prothorax when *Scr* is knocked down. Our previous work in *Oncopeltus* showed that *Scr*-RNAi adults have both ectopic winglets and an ectopic scutellum on the T1 segment (Chesebro et al., 2009), suggesting that *Scr* represses both of these structures in wild type. The
transcriptome analysis of these tissues (Ch.3) provides evidence that the T1 ectopic wings and scutellum are comprised of cells from strictly a dorsal component, lacking any ventral input. If so, this new insight now allows us to examine what elements are involved in shaping the dorsal component of the wing program. Therefore, further studies of Scr can be used to tease apart its dual function (suppression of both the wings and scutellum) in the prothorax and to better understand the origins of dorsal appendages and structures on the insect thorax.

To initiate these studies, we must first understand the events that give rise to a true wing (which are diagrammed in Figure 4.1A). During larval development different compartments are specified depending on the presence or absence of various morphogens. The dorsal/ventral (DV) compartments are determined by the presence or absence of the localized expression of *engrailed*, while expression or absence of *apterous* determines the anterior/posterior (AP) compartments (Neto-Silva et al., 2009). Cells in the anterior and posterior compartment communicate through Hedgehog signaling which leads to the expression of *decapentaplegic* (*dpp*) in the anterior cells near the compartment boundary of the wing disc. The presence of *ap* in cells along the DV boundary leads to the activation of Notch (N) signaling and induces *wg* and the *vestigial* boundary enhancer (*vgBE*) (Couso et al., 1995; de Celis et al., 1996; Diaz-Benjumea and Cohen, 1995; Kim et al., 1996). The combined activity of Dpp and Wg in the developing wing disc controls cell fate and growth by inducing *vestigial* (*vg*) which is necessary for wing identity (Kim et al., 1996; Klein and Arias, 1999; Williams et al., 1991). *vg* may indirectly induce expression of *nubbin* (*nub*) (del Álamo Rodríguez et al., 2002) which is the earliest marker for wing fate to be expressed in the presumptive wing
field (Ng et al., 1995; Ng et al., 1996). Wg activity represses *homothorax* (*hth*) and *teashirt* (*tsh*) and it is the repression of the latter that is important in establishing distal wing fate (Casares and Mann, 2000). Repression of *hth* in the distal regions of the wing is also important but is considered a secondary event in determining wing fate (Wu and Cohen, 2002).

While the wing pouch is dependent on the activity of Wg, the notum (dorsal plate) is determined by the EGFR signaling pathway. In the proximal cells *vein* (*vn*) activates EGFR, which in turn induces *Iroquis-complex* (*Iro-C*) genes. The *Iro-C* genes specify cells that will become notum, while those cells in which *Iro-C* gene are knocked down will acquire a hinge identity (Cavodeassi et al., 1999; McNeill et al., 1997; Wang et al., 2000; Zecca and Struhl, 2002, 2007). The distal border of *Iro-C* is established by Muscle segment homeodomain (Msh), which is restricted to the hinge region only, and Dpp (Cavodeassi et al., 2002; Villa-Cuesta and Modolell, 2005). As illustrated in Fig. 4.1C, *nubbin* is expressed both in the wing pouch and in the distal hinge along with *rotund* (*rn*) (del Álamo Rodríguez et al., 2002; St Pierre et al., 2002).

The hinge is divided into three mutually exclusive growth domains: the distal domain containing *nub* expressing cells (light blue in Fig. 4.2), the proximal domain containing *tsh* expressing cells (yellow in Fig. 4.2), and a gap domain (grey in Fig. 4.2) between the two which contains cells that do not express either *nub* or *tsh* (Zirin and Mann, 2007). All three domains are dependent on *wg* signaling. Initially the *nub* and *tsh* expressing cells are abutted against one another, and the interface between them is made of *wg* expressing cells (pink outlined cells in Fig. 4.2). These *wg*-expressing cells “push” the *tsh* expressing cells away from the *nub* expressing cells creating a region
lacking either gene, which gives rise to the medial region of the hinge (grey in Fig. 4.2). As shown in Fig. 4.1C, the cells of the Wingless outer ring (Wg OR) are maintained by a combination of \textit{hth} and \textit{tsh} and give rise to the proximal hinge region (Ng et al., 1995; Zirin and Mann, 2007). Similarly, the cells of the Wingless inner ring (Wg IR) are maintained by \textit{hth}, and together with \textit{nub} define the distal hinge (Azpiazu and Morata, 2000). Hence, the wing developmental pathway is the culmination of both \textit{wg} and \textit{dpp} signaling that jointly reshape the T2/T3 thoracic segments resulting in a wing being separated from the notum by a hinge.

In order to begin to understand how these previously described wing genes function together in the re-modeling of the T1 notum, we will first focus on the roles of \textit{vg} (the wing selector gene) and \textit{nub} (one of the first wing initiation genes). This can be accomplished by examining these genes functions in the T1 plate of \textit{Scr} RNAi individuals, when the wing program is re-initiated. Note that in this particular instance, the resulting structures (ectopic wings and scutellum) represent only the dorsal elements of the wing program (as documented by the transcriptome data analysis in Chapter 3). Therefore, by examining the functions of \textit{vg} and \textit{nub} in these insects we can begin to investigate their role(s) in shaping the dorsal component of the wing program in the absence of any ventral contribution normally found in T2/T3 wings. In a complementary analysis, we will also examine the connecting structure between the wing and notum, namely the hinge. For this purpose we will determine the function of \textit{tiptop (tio)}, the \textit{Oncopeltus} ortholog of \textit{teashirt}, which was shown to shape the hinge and adjacent tissue in \textit{Drosophila} (Soanes and Bell, 2001; Soanes et al., 2001; Wu and Cohen, 2002; Zirin and Mann, 2007).
Figure 4.2. Establishment of *nub* and *tsh* boundaries as related to notum, wings and hinge determination. This drawing is based on Zirin and Mann (2007).
Results and Discussion

As shown in the previous chapter, the prothorax normally features a broad and flattened morphology (Figure 4.3A), and in Scr RNAi individuals takes on characteristics of the dorsal T2 segment with both an ectopic scutellum and wing vestiges (Figure 4.3B-B’). Close examination of the T1 plates in Scr RNAi adults showed that their overall length is very similar to wild type, indicating that the presence of the ectopic scutellum in the former is not caused by extra tissue growth. Rather, it seems that the observed RNAi-caused alterations are the result of the re-modeling of the T1 notum in which an invagination in its posterior half causes a separation between the scutellum and ectopic wings (asterisk in Figure 4.3B). This separate, but coordinated development of notum (scutellum) and wing is one of the events that occur during the development of the wing program. As described in Chapter 3, the ectopic wings on T1 in Scr RNAi individuals are comprised of only dorsal components of the wing program. Therefore, these appendages provide a unique opportunity to tease apart the function of key wing genes in the dorsal wing program. This can be accomplished by performing a set of double RNAi experiments, with one gene being Scr (to reinitiate the wing program in T1) and the other one being a conserved wing gene (to assess its function in the development of the dorsal wing compartment).

First, we performed Scr/vg RNAi, and all of the resulting individuals lacked both the ectopic wings and scutellum (Figure 4.3D). This result is consistent with Drosophila studies where the loss of vg, the main wing initiation gene, results in a near complete loss of wings (Williams et al., 1991). Also, in agreement with our results in Fig. 3.4, the depletion of vg is also sufficient to prevent the formation of the ectopic scutellum. In our
second double RNAi experiment, we depleted both the Scr and nub. In these Scr/nub RNAi individuals, while ectopic wings do not develop, there is a thickening of the tissue along the lateral edges of the T1 plate, which may represent wing precursors (Figure 4.3F-F’). This means that although the wing program has been stopped prematurely, it had progressed further than the vg RNAi phenotype. In contrast to the situation with wings, the ectopic scutellum does form in Scr/nub RNAi adults. However, it is not very pronounced suggesting that nub does not play a role in its development. These observations are consistent with our previous results of nub expression in T2 wing pads (Fig. 3.4) that showed its presence only in the wing primordia, and not in the scutellum. The results from the Scr/vg experiment suggest that the wings and scutellum are part of an integrated wing program since the knockdown of the wing “master” gene vg results in the loss of both the wings and scutellum. Furthermore, the scutellum is likely not created independently of the wings but may be the result of their coordinated development (Fig. 4.3F), where the initiation of wings results in the beginnings of a scutellum. Taken together these results indicate that the dorsal wing program is responsible for the remodeling of the notum, with the end result being the creation of a scutellum that is separate from flattened tissue along the lateral margins.

Based on the above results we can hypothesize that Scr acts upstream of vg and nub and inhibits the functions of these gene products. Furthermore, comparison of single vg or nub RNAi effects on T1 plates with those of Scr/vg and Scr/nub RNAi, reveals the presence of a ridge that separates the anterior and posterior regions of the plate in double depletions (white arrowhead in Figs. 4.3D and 4.3F). This is reminiscent of the supernumerary segment seen in Periplaneta Scr RNAI individuals, suggesting
Figure 4.3. Effects of *Scr*, *vg*, and *nub* RNAi on the T1 morphology. (A-F) Dorsal and (A’-F’) frontal view of wild type T1 plate (A and A’), *Scr* RNAi T1 plate (B and B’), *vg* RNAi T1 plate (C and C’), *Scr/vg* RNAi T1 plate (D and D’), *nub* RNAi T1 plate (E and E’) and *Scr/nub* RNAi T1 plate (F and F’). Asterisks show separation of notum and wing primordia while white arrowheads denote a ridge forming in the T1 plates of *Scr/vg* and *Scr/nub* RNAi individuals.
that Scr may alter *engrailed* (*en*) expression (Hrycaj et al., 2010) and thereby disrupt the signaling along the anterior-posterior axis of the wing program. This, in turn, would cause alterations of *dpp* signaling which is necessary for proper wing development (please refer to Fig 4.1A for possible interactions between *en* and *dpp*).

In *Drosophila*, both *wg* and *dpp* are required to activate *vg*, which in turn represses *hth*. Consequently, the initial expression of *hth* throughout the wing pouch is abolished. At low levels of *dpp*, *vg* is not activated and *hth* is not causing the wing pouch to assume a notum identity repressed (Zirin and Mann, 2004). Also, the expression of Iro-C genes, normally restricted to the notum, extends to the distal limit of the *tsh* domain at low levels of Dpp. This results in the loss of the hinge structure and the fusion of the notum and wing. Therefore, Dpp signaling is thought to be essential for the separation of wing and body wall (Cavodeassi et al., 2002). These observations indicate that low levels of *dpp* cause a loss of separation between these two structures, which consequently leads to the entire wing disc taking on the identity of the notum due to expression of body wall genes. In the T1 plate of *Scr* RNAi adults, the notum is separated from the ectopic wings (asterisk in Figure 4.3B) suggesting an increase in *dpp* levels in this region. Therefore in wild type, *Scr* may be involved in decreasing *dpp* levels, either directly or indirectly. Our transcriptome data support this hypothesis, as it shows that *dpp* and other members of its signaling pathway expanded (*ex*), bunched (*bun*) and division abnormally delayed (*dally*) are upregulated in *Scr* RNAi T1 as well.

While the explanation above may explain how *Scr* functions to remodel the T1 notum, the development of the hinge and adjacent tissue also needs to be addressed. In wild type *Oncopeltus* the wings lay flat against the body (Fig. 4.4A), and they display
a tight fit against the scutellum (Fig 4.4B). In tio RNAi individuals, the wings no longer rest against the body (Fig. 4.4G) and the scutellum is altered and assumes a shield-like shape (Fig. 4.4H). While the overall size and shape of fore- and hindwings are not altered (compare Fig. 4.4D and F with 4.4J and L), the main effect can be seen in the hinge region. As illustrated in Fig. 4.4C, there is a specific structure, sclerite (black arrowhead) in the anterior region of the hinge in wild type. Additionally, there is a membrane in the posterior hinge region that connects the wing to the notum (white arrow, Fig. 4.4C). In tio RNAi adults, however, the sclerite is reduced or completely absent (Fig. 4.4I). In addition, the membrane that connects the wing to the notum is no longer present causing a partial fusion between the two structures. A similar trend is observed in the anterior region of the hindwing. In wild type, there is a structure that aids in its articulation and support (black arrowhead Fig. 4.4E). Also, the anal lobe separates this wing from the notum in the posterior region. In tio RNAi the supportive and articulating structure in T3 is reduced or absent (black arrowhead Fig. 4.4K) and the anal lobe is now fused to the notum similar to that seen in the T2. The lack of separation of the wings from the notum is consistent with studies in Drosophila in which mutations in tsh (a tio ortholog in flies) cause a fusion of wings to the notum, which in turn, results in wings being outstretched from the thorax (Soanes and Bell, 2001; Soanes et al., 2001).

The altered morphology in tio RNAi Oncopeltus adults (Fig. 4.4G) that is described above mirrors Drosophila mutant tsh phenotypes, suggesting that it may be possible to use the Drosophila paradigm of hinge development to explain these phenotypes. As shown in Fig. 4.1C, in Drosophila both the Wg IR and Wg OR are
Figure 4.4. Effects of tio RNAi on adult morphology. (A) Wild-type adult *Oncopeltus* showing the wings folded flat on the body. (B) Close-up of wild-type scutellum. (C) Close-up of wild-type T2 hinge showing the sclerite (black arrowhead) and connecting membrane (white arrow) and (D) Wild-type forewing. (E) Close-up of wild-type T3 hinge and (F) hindwing. (G) tio RNAi adult showing the misfolding wings. (H) Close-up of tio RNAi scutellum. (I) Close-up of tio RNAi T2 hinge and (J) forewing. (K) Close-up of tio RNAi T3 hinge and (L) hindwing.
required to maintain a hinge identity. Disruption of either Wg IR or Wg OR results in the loss of Msh expression, which causes nub expressing cells (wing specific) to abut hth expressing cells (notum specific). In the absence of tsh (tio orthologue in flies), the formation of Wg OR is disrupted (since tsh is required for maintaining Wg OR expression) and we would expect the wing to be fused to the notum. Based on these insights from Drosophila, we would expect a similar phenotype when tio is knocked down in Oncopeltus. As illustrated in Fig. 4.4I and K this prediction is indeed confirmed, suggesting that tio (tsh) plays a similar role in both Drosophila and Oncopeltus. While its loss causes the fusion of the wings to the notum, it is important to recognize that the overall morphology of the wings is unchanged in both species (Fig. 4.4J and L, (Soanes and Bell, 2001; Soanes et al., 2001)). Therefore, our results suggest that the hinge is formed independent of the wings. Furthermore, the formation of the hinge is closely associated with the development of the scutellum in Oncopeltus. These findings provide a new insight into the genetic mechanisms regarding the co-development of the scutellum and hinge. Consequently, we may now be able to begin to understand how the interplay of signaling events involved in the development of the wing, scutellum and hinge allowed for the transition from paleopteran (outstretched wings) to neopteran (wings folded flat) insects.

**Material and Methods**

**Cloning and sequence analysis of tiptop (tio)**

Mixed stage Oncopeltus fasciatus embryos were used for total RNA extraction. The production of cDNA, RT-PCR and cloning were performed according to (Li and
Popadic, 2004). A 300 bp fragment was amplified using the forward primer 5’ GCT GAA CAC GCG GCA GAT T and the reverse primer 5’ AGT CAA AAC CGC GCT GAC ATG. Ten clones were recovered, sequenced and compared with each other as well as the existing Oncopeltus tio gene (GenBank accession number AF533539). For Scr and nub previously characterized fragments were used (Chesebro et al., 2009; Hrycaj et al., 2008), for vg the fragment described in Chapter 3 was used.

**RNA interference (RNAi)**

Double stranded RNA (dsRNA) was prepared and injected into the abdomens of Oncopeltus nymphs, as described by Chesebro et al. (2009). For Scr, vg, nub, Scr/nub and Scr/vg RNAi approximately 2µl of dsRNA was injected at a concentration of 2µg/µl into Oncopeltus 4th and 5th nymph consecutively, a total of 15 nymphs were injected for each set of injection of which 7 Scr/vg and 8 Scr/nub RNAi individuals molted into adults. For tio RNAi, 15 Oncopeltus 5th nymphs were injected with approximately 2µl of dsRNA at a concentration of 2µg/µl of which 8 individuals molted into adults.

**Microscopy and Image Analysis**

Images were taken using an Olympus SZX16 microscope and Olympus DP72 camera.
APPENDIX

APPENDIX 1

The dissected fore- and hindwings of *Apis mellifera* and expression pattern of UbdA in their respective wing discs. A. The adult forewing morphology. B. The T2 larval wing disc shows the absence of UbdA expression. C. The adult hindwing morphology. D. In contrast to forewings, there is a high level of UbdA expression throughout the T3 larval wing disc.
APPENDIX 2

Effects of Ubx RNAi on Apis fore- and hindwings. A. Wild type adult T2 wing. B. Ubx RNAi forewing that shows no effect in its morphology. C. Wild type adult T3 wing, inset shows the specific venation pattern. D-D'. Ubx RNAi T3 wing, insets now illustrate the changes in its venation.
apterous (*ap*) *in situ* staining of *Oncopeltus* 5th nymph wing pads. A. The forewing shows *ap* expression that is mainly localized in the proximal half of the wing pad. B. In contrast, the hindwing shows almost complete absence of *ap* expression.
APPENDIX 4

*Ubx in situ* staining of *Oncopeltus* 5th nymph wing pads. A. The forewing shows a ubiquitously distributed, very low levels of *Ubx* (likely due to background staining). B. The hindwing shows strong expression of *Ubx* throughout the entire wing pad. C-D. Sense controls showing virtually no expression in either forewing or hindwing.
**APPENDIX 5**

*homothorax (hth)* *in situ* staining of wild type and *nub* RNAi 5th nymph wing pads. A-B. The fore- and hindwing pads show no expression of *hth*. C-D. The sense *hth* RNA probe shows no expression in either wing. E. The *nub* RNAi forewing shows that the expression of *hth* can now be observed throughout the entire wing pad.
APPENDIX 6

*nub in situ* staining of *Oncopeltus* 5th nymph wing pads. A-B. The fore- and hindwings show strong expression of *nub* throughout the entire wing pad. C-D. The sense controls showing no expression in either wing.
APPENDIX 7

wingless (wg) in situ staining of Oncopeltus 5th nymph wing pads. A-B. The fore- and hindwing shows wg expression only along the outer edges of the wing pad (denoted by arrowheads). Expression seen in the wing veins is likely due to background staining. C-D. The sense controls show no expression.
APPENDIX 8

Effects of *vestigial (vg)* and *scalloped (sd)* RNAi on mitotically active cells. A-B. The wild-type fore- and hindwings show actively dividing cells as seen by the punctated green signal of anti-histone 3 (green dots), which can be used to identify actively dividing cells. C-D. The fore- and hindwings of vg RNAi 5th nymphs show a decrease in the number of actively dividing cells. E-F. Similarly, the fore- and hindwing of sd RNAi 5th nymphs also show a decrease in actively dividing cells. Note that the broad regions displaying light green color represent staining artifacts.
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Insects display the greatest amount of structural and functional variation among animal groups, particularly in regard to their appendage morphology. These differences can range from the diverse pigmentation patterns between fore- and hindwings to changes in the size and shape of legs. The greatly enlarged jumping hind leg in crickets and grasshoppers is one of the best known illustrations of such diversity, representing a unique feature for the entire order of these insects (Orthoptera). Previous work from our lab has shown that the homeotic gene Ultrabithorax (Ubx) plays a key role in the enlargement of hind legs not only in orthopterans, but other insect species as well. Another example of a greatly modified hind leg is seen in the honeybee, Apis mellifera, which has been adapted for the collection and carrying of pollen. To determine if these modifications are also regulated by Ubx, we used the RNAi approach to examine its function in the developing Apis legs. Our results show that Ubx is indeed responsible for the formation of the pollen basket and its associated structures, and is also expressed in the same region in another bee species, Bombus impatiens. These results indicate that this gene likely has a conserved role in the
formation of the pollen basket, a unique structure common to all social bees. Therefore, 
*Ubx* may not only responsible for the enlargement, but also for the specific 
morphological specialization of the T3 leg in insects.

In addition to legs, wings are another set of appendages that display a wide 
range of morphological diversity. They also represent a novel adaptation that allowed 
for the rapid and highly successful radiation of insects into almost every ecological niche 
on Earth. Hence, to better understand the evolution of insect wings we must elucidate 
both the origins and divergence of these structures. Here we use an unbiased global 
transcriptome analysis combined with a candidate gene approach to begin to answer 
these questions. Our data show that knocking down wing genes affects specific regions 
in the prothorax (T1 segment), which may be considered to be wing serial homologs. 
This is the first evidence that such structures were identified in hemimetabolous insects. 
Furthermore, the depletion of the same wing genes (by RNAi) also plays a role in 
shaping the scutellum, the dorsal plate on the second thoracic (T2) segment. This 
result suggests that the wings and dorsal plates may have co-evolved, which in turn, 
may have enabled proper wing folding. Finally, our data reveal that the fore- and 
hindwings are composed of both dorsal and ventral components confirming the 
predictions of the recently proposed combinatorial model. However, the ectopic wings 
on T1 were found to lack any ventral contribution and constitute strictly a dorsal wing 
program. This may explain why fossil hemipteroids featured underdeveloped T1 wings, 
leading to their ultimate loss in the lineages giving rise to modern-day insects. Overall, 
the present work provides several new insights that further our understanding of wing
origins and divergence and help establish a robust and testable framework for future studies of wing evolution.
I completed my Bachelor of Science (B.Sc.) degree at the University of Windsor in Windsor, Ontario Canada. After the completion of my degree I decided to spend some time abroad to learn more about my heritage and moved to Croatia. Upon my return I was employed at the Center for Molecular Medicine and Genetics at Wayne State University School of Medicine as a Lab Technician working with Human Papillomavirus (HPV). It was during this time that I discovered how much I enjoy research and that I wanted to pursue a career in research. I initially enrolled in the Master's of Biotechnology at Wayne State University, but soon realized I wanted to be more than a technician so I transferred to the PhD program. I joined the Popadić lab because of my interest in developmental biology. My work concentrated on the origin and development of insect wings, a question that has been debated for decades. In my time at Wayne State University, I have been able to meet my goals of publishing original work as well as teaching undergraduate students both in the lab and in the classroom. During my time as a graduate teaching assistant, I received the Graduate Teaching Award three times (2009, 2011 and 2013).