Cellular and Molecular Patterning Mechanisms Underlying Metamorphosis of the Thoracic Leg in *Manduca sexta*

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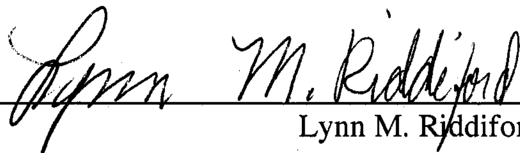

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Abstract

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Metamorphosis allows insects to possess radically different morphologies adapted for larval and adult functions. The tobacco hornworm *Manduca sexta,* like most other insects with complete metamorphosis, makes two versions of thoracic legs. The simple thoracic legs of the larva are formed during embryogenesis. During metamorphosis, the leg goes through extensive remodeling to give rise to the adult leg with a drastically different morphology. In addition, the adult legs on each of the three thoracic segments develop segment-specific features not present in the larval legs. In this dissertation, I explore the molecular and cellular mechanisms underlying three aspects of this morphological transformation.

First I investigated the relationship between the cells in the larval and adult legs. The larval leg epidermis was found to consist of three distinct cell populations in terms of their contribution to the adult leg. The first population undergoes a rapid proliferation during the last larval instar to make up majority of the adult leg. The second population goes through no or very little proliferation and contributes to small parts of the adult leg. The last population is eliminated by programmed cell death during metamorphosis.

Next, expression patterns of five leg patterning genes were examined to study their role in generating the two versions of the leg. I found that the adult leg of *Manduca* is produced by the conserved expression pattern of these genes that produce the adult legs

of other insects. The larval leg, on the other hand, was produced by interruption of this adult patterning process. Completion of the patterning process at metamorphosis involved *de novo* expression of these genes in some populations of cells.

In the last study, I examined the role of the Hox gene *Sex combs reduced (Scr)* in the segment-specific differentiation of the first thoracic (T1) legs during metamorphosis. During the early and late embryonic development, Scr protein was expressed weakly throughout the T1 leg. During the pupal leg development, its expression was up-regulated in regions of the T1 leg that develop segment-specific features consistent with its role in segment-specific development of T1 leg at this stage.

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DEDICATION

To all the leggy creatures on the planet, who have always tweaked my curiosity.

Chapter 1
Introduction

Metamorphosis is a postembryonic event in which one stage of an animal undergoes a radical morphological transformation, thereby accommodating the functional and ecological demands of the next stage. Many groups in variety of phyla employ this effective life history strategy (Strathmann 1993; Wray 2000). Some groups have more than two distinct postembryonic stages that are disparate in form and function (e.g. heteromorphic insects, barnacles etc). Despite our fascination with this phenomenon for centuries (Hall and Wake 1999; Erezyilmaz 2004a), we still have very little idea about how instructions for making two (or more) disparate body forms are encoded in and executed from a single genome. There has been a large body of work over the last half century examining the developmental "switches" from the larval to the adult developmental programs (Nijhout 1994; Rose 1999; Heyland et al. 2004). However, understanding how an animal's genome activates developmental genes to generate multiple body forms has only recently become possible, as the fields of molecular and developmental genetics have elucidated the molecular patterning mechanisms that produce animal forms.

In insects, the transition between the larval and the adult forms is mainly due to three organismal-level phenomena. First is the heterochronic delay in development of adult-specific features such as wings and genitalia to the time of adult development. Second, the larval-specific features such as the abdominal prolegs, defensive hairs and spines are discarded at metamorphosis. Lastly, the organs that are functional in both the larval and adult stages, such as mouthparts and legs, go through radical remodeling from the larval to the adult form (Truman and Riddiford 1999, 2002).

In order to study the developmental basis of the last phenomenon, I focused on the metamorphosis of thoracic legs. The insect leg is an excellent model to investigate how a single organ generates two forms both in ontogeny and phylogeny. First, the development and molecular patterning of insect legs is one of the best studied systems,

thanks to an extensive body of work in the model system *Drosophila melanogaster* (Kojima 2004; Fristrom and Fristrom 1993). Secondly, we know that the same set of genes found in *Drosophila* is also used in leg development of other insects and arthropods (Angelini and Kaufman 2004). Thirdly, there is a rich body of studies, both classic and current, on molecular and physiological mechanisms controlling metamorphosis (Riddiford et al. 2003). Finally, in insect evolution, we know that a metamorphic life cycle evolved from a non-metamorphic ancestor and the non-metamorphic insects are still present to be used for comparative developmental studies (Truman and Riddiford 1999, 2002). This is not the situation for many of the other animal groups where the ancestry of metamorphic life cycles is uncertain (Strathmann 1993).

Insect leg development can be categorized into three major types. The first is the basal mode observed in the ametabolous and hemimetabolous insects. In this mode of leg development, the adult-like legs are produced during embryogenesis. These legs simply grow in size during the nymphal stages. The second mode of leg development is basal to the holometabola. Simple larval legs are formed during embryogenesis and these legs are transformed into more complex adult legs during metamorphosis. The last mode of leg development is unique to larvae that have lost their thoracic legs. In these insects, the adult legs grow inside the larval body as imaginal discs. During metamorphosis, these discs evert to form the adult legs.

The tobacco hornworm, *Manduca sexta,* exhibits the second mode of leg development. The simple larval leg is produced during embryogenesis. This leg consists of the five primary segments common to all insect legs, but it is only capable of grasping motions (Kent et al. 1996). During metamorphosis, this leg goes through extensive remodeling to give rise to the adult leg with drastically different morphology. The adult leg is about seven times longer than the larval leg and each segment has gone through differential growth. The distal-most segment, the tarsus, is divided into five smaller subsegments. Also, the legs on each of the three thoracic segments develop unique structures such as an antenna-cleaning organ on the first thoracic leg and spurs on the second and third thoracic legs.

In this thesis, I explore the molecular and cellular mechanisms underlying three aspects of this remarkable transformation from the larval leg into that of the adult. First, I investigated the relationship between the cells in the larval and the adult legs. My results showed that the larval leg epidermis consists of three distinct cell populations in terms of their contribution to the adult leg. The first population undergoes a rapid proliferation during the last larval instar to make up majority of the adult leg. The second population goes through no or very little proliferation and contributes to small parts of the adult leg. The last population is eliminated by programmed cell death during the prepupal stage. Having established that, I then examined expression patterns of five leg patterning genes to study their roles in generating the two versions of the leg. I found that the remodeling of the leg involves changes in the expression patterns of these genes and conclude that the larval leg is produced by interruption of the adult leg patterning process. In the last study, the molecular basis of the segment-specific differentiation of the thoracic legs during metamorphosis was explored. Specifically, I have examined the expression pattern of the Hox gene *Sex combs reduced*, which directs development of morphologies specific to first thoracic legs in insects. In *Manduca*, its expression was up-regulated in regions of the first thoracic leg that go through segment-specific development during metamorphosis. I use the concluding chapter to bring the three aspects of leg metamorphosis together to discuss development and evolution of larval forms in insects.

Chapter 2

Development of the adult leg epidermis in *Manduca sexta*: contribution of different larval cell populations

Introduction

Metamorphosis in insects allows the larval and the adult stages to possess radically different morphologies adapted to their respective environments and functions. Some organs such as wings and genitalia are only required for the adult stage. Thus, their development can be initiated at any time before the adult development and their final form then achieved during metamorphosis. Other organs, by contrast, have to be functional in both stages. In such cases, one version is produced during embryogenesis to fit the larval needs, but then during metamorphosis the organ has to be remodeled to a form appropriate for the adult. The cellular and molecular patterning mechanisms that sequentially generate these divergent organ forms are not well studied. In order to gain insight into this type of development, I investigated the leg development in the tobacco hornworm *Manduca sexta*.

The larval leg of *Manduca* is a simple structure only capable of lateral motion (Kent et al. 1996), although it already has the five primary segments characteristic of the adult insect legs (Fig. 2.2a). All the epidermal cells in this leg make the larval cuticle during the larval molts. However, in the beginning of the final larval instar, the adult primordia, a small subset of these cells occupying a longitudinal dorsal strip and circumferential rings near the joints (Fig. 2.1), detach from the cuticle and initiate rapid proliferation (Booker and Miles 1993). This proliferative process results in morphogenesis and growth of the more complex adult leg, which is about seven times the length of the larval structure (Fig. 2.2b). Each segment goes through differential growth to attain a unique morphology. In addition, the tarsus, which consisted of one segment in the larva, becomes subdivided into five segments in the adult.

Questions regarding the relationship between the larval and the adult legs have been addressed in a few lepidopteran species including *Manduca.* Some studies have attempted to establish homology between the segments in the larval and the adult legs using morphological observations, surgical experiments and a mutant strain with leg phenotypes (Kim 1959; Bodenstein 1949; Miles and Booker 1993; Birket-Smith 1984). At the cellular level, relationships between the muscle cells, sensory and motor neurons of the larval and the adult legs have been examined in great detail in *Manduca* (Consoulas et al. 1997; Consoulas 2000; Kent and Levine 1988).

Although the most visible difference between the larval and the adult legs of *Manduca* and other lepidopterans is due to the transformation of the epidermis, no study has investigated the relationship between the epidermal cells of the two legs. It has simply been assumed that the cells in the adult primordia give rise to all or most of the adult leg epidermis, while the cells in the regions outside, which occupy most of the larval leg, either degenerate (e.g. Kent and Levine 1988; Consoulas 2000; Miles and Booker 1993) or make minimal contributions to the adult structure (Truman and Riddiford 2002). An answer to this unresolved question has several important implications. Firstly, it will tell us whether there is a distinct cell population that is terminally differentiated for larval leg function. Secondly, whether and how much of the regions outside the adult primordia participate in the adult leg formation will present a very different starting condition for the molecular patterning of the adult leg. Finally, knowing the relationship between the cells in the larval and the adult leg epidermis will tell us how the leg development via an imaginal disc may have evolved in more derived insects such as *Drosophila melanogaster.*

In this chapter, I address the above question by labeling the cells outside the adult primordia with bromodeoxyuridine and following their fate during the adult leg development. I also characterize patterns of cell proliferation and cell death during early adult leg morphogenesis. I show that some larval cells outside the adult primordia survive to make small parts of the adult leg. However, most cells in these regions are eliminated through apoptosis. My results will be discussed in context of the homology

between the larval and the adult leg segments and adult leg development in other holometabolous insects.

Materials and Methods

Experimental animals

Larvae of *Manduca sexta* (L) were raised in individual containers on an artificial diet (Bell and Joachim 1976) at 26°C under a long-day photoperiod (17hr light and 7 hr dark). After they initiated wandering, larvae were placed in holes bored in wooden blocks for pupation. The animals were chilled on ice before dissection or injection.

The developmental stages of animals were determined with reference to developmental transitions. The day of ecdysis to the last larval instar was designated V+0. A day after V+3, larvae began to wander (W+0). Wandering stage lasted approximately 4 days after which the animals pupate (P+0).

Dissections

The ventral thorax with the thoracic legs was spread and pinned down onto a Sylgard-lined Petri dish in *Manduca* saline (Riddiford et al. 1979). Muscles, fat body and tracheae were removed and the tissue fixed in 3.7% formaldehyde in phosphate-buffered saline, pH 7.2 (PBS; 130mM NaCl, 7mM Na_2HPO_4, 3mM NaH_2HPO_4) for 2 hr at room temperature or overnight at 4°C, followed by rinses in PBS with 1% Triton-X 100 (PBS-TX; Sigma, St Louis, MO). To expose the leg epidermis still attached to the leg cuticle (V+0 through W+1), a sharp razor blade was used to split open individual legs into either dorsal/ventral or anterior/posterior halves. The nerves, tracheae, muscles and fat body were removed to expose the epidermis. For the later stages (after W+2), the leg epidermis could be carefully removed from the larval or pupal leg cuticle.

Detection of proliferating cells

Epidermal cells going through proliferation between W+1 to W+3 were identified by their incorporation of a pulse of 5-bromodeoxyuridine (BUdR) (Gratzner 1982; Sigma, St Louis, MO). Animals were anesthetized on ice and injected into the abdomen with 20μg/μl BUdR in *Manduca* saline (15μl/g body mass) using a 100μl Hamilton syringe (Hamilton Company, Reno, NV). After 6 hr, the legs were fixed and dissected as above. Fixed tissues were acid-hydrolyzed in 2N HCl in PBS-TX for 1 hr. After a few rinses, they were blocked in 5% donkey serum in PBS-TX for 25 min. BUdR was detected with 1:200 anti-BUdR antibody (Becton Dickinson Immunocytometry Systems, San Jose, CA) followed by 1:1000 Alexa Fluor 488 goat anti-mouse antibody (Molecular Probes, Eugene, OR). Legs were treated with 100 μg/ml RNAse for 30 min, counterstained with 1:200 0.4 mg/ml propidium iodide to visualize the nuclei, then dehydrated, cleared in xylene, and mounted in DPX (Fluka, Buchs, Switzerland).

Cell labeling using BUdR

BUdR incorporation was also used to follow the fate of cells outside the adult primordia. A BUdR stock solution (10 mg/ml BUdR in 40% EtOH) was mixed into the artificial diet melted in a microwave. Concentrations of 0.1, 0.2, 0.3 and 0.5 mg/ml were tested, and 0.2 and 0.3 mg/ml were found to be the optimal concentrations for survival and labeling. Larvae were fed the BUdR diet from hatching until the end of the penultimate (4th) larval instar. At the start of the 5th larval instar, they were switched to the regular diet free of BUdR. During the 5th instar, rapidly dividing cells then lost their BUdR label. Larvae were dissected and processed at various stages for BUdR immunocytochemistry as described above.

Cell death detection

Incorporation of Acridine Orange (AO; Sigma, St. Louis, MO) was used to characterize location and timing of cell death. Legs were dissected in *Manduca* saline and incubated in 1 μg/ml AO in *Manduca* saline for 5 min, followed by 5 quick rinses

with *Manduca* saline. The tissues were mounted in then *Manduca* saline under a coverslip with plasticine spacers and imaged using fluorescence or confocal microscopy.

Two other techniques were used to confirm the AO results. For the TUNEL (terminal deoxynucleotidyl transferase mediated dUTP nick end labeling) (Gravrieli et al. 1992) assay, tissues were fixed for 2 hr in 3.7% formaldehyde in PBS followed by several washes in PBS-TX. They were then rinsed three times in 10 mM Tris, pH 7.5 and incubated in 20 μg/ml Proteinase K in 10 mM Tris, pH 7.5 for 1 hr at 37°C. The reaction was stopped by washing three times in PBS-TX. *In Situ* Death Detection Kit Fluorescein (Roche Applied Science, Indianapolis, IN) was used for the TUNEL reaction. The tissues were equilibrated in TdT buffer and the reaction carried out as in the protocol. The tissues were washed five times in PBS-TX, then twice in PBS and mounted as above. Observation of the positive control, in which the legs were incubated with 10 U/ml DNAse for 30 min at 37°C to fragment DNA of all the cells, indicated that only the DNA in the epidermal cells was accessible to the labeling reaction with our whole mount method.

Lastly, pyknotic nuclei were detected by staining with propidium iodide. These highly condensed nuclei, a feature of apoptotic cells, appeared compact and brighter compared to normal nuclei. The legs were fixed and stained with propidium iodide as above. The location and the timing of appearance of pyknotic nuclei were identified using confocal microscopy.

Image collection and analysis

All the fluorescent images were collected using the confocal microscope. Since I was interested in the epidermis, non-epidermal areas were removed from each confocal section using the lasso tool in the public domain NIH Image program (developed at the U.S. National Institutes of Health). To assemble images of the whole leg, image stacks from each leg region were processed through Z-series projection in NIH Image and the individual pieces assembled into a montage using Adobe Photoshop (Adobe Systems Inc., San Jose, CA). Most of the legs and all the legs older than mid

W+2 were from the metathoracic (T3) segment. The T3 leg has two pairs of spurs on the tibia, which became visible around mid W+2, and provided convenient positional markers (Figs. 2,5).

Results

Adult leg morphogenesis

Adult leg development in *Manduca* begins inside the larval leg cuticle. About 24 hours after the molt to the final larval instar, the cells of the adult primordia (Fig. 2.1), which were a part of the larval leg epidermis, detach from the larval cuticle and begin rapid proliferation (Miles and Booker 1993). The location of the primordia and the order in which they initiate proliferation are similar to those described in another Lepidoptera, *Pieris brassicae,* by Kim (1959) (Miles and Booker 1993; unpublished data; the adult primordium is referred to as "differentiation center" by other researchers). The adult primordia grow as prominent invaginations inside the larval leg cuticle during the last larval instar (Figs. 1, 2c). The areas outside of the adult primordia, however, remain attached to the leg epidermis. In this paper, I refer to these areas as the larval regions since they occupy most of the larval leg epidermis (Figs. 1, 2c). Since the larval regions are associated with the giant bristle cells and the muscle attachments, they must remain attached to the cuticle to maintain their function during the last larval instar. On W+2, they finally detach from the cuticle, allowing the growing leg to be removed from the larval leg cuticle (Fig. 2.2d-h). Bu that time, the areas corresponding to the adult primordia are evident as highly folded regions, while the larval regions are recognized by the persistence of the large bristle nuclei (Fig. 2.2d). The leg grows in length both due to elongation of the folded regions and continued cell proliferation. As the morphogenesis proceeds the lobe-shaped ventral tibia is lost (Fig. 2.2d, e) and the two pairs of tibial spurs appear by early W+3 (Fig.

2.2g). By mid W+3 (Fig. 2.2h), the overall morphology of the adult leg is attained, although further growth and additional morphogenesis take place until the mid pupal stage. Both the proportion and the actual size of the larval regions decrease significantly, but they are still discernible at W+3 as regions associated with the giant bristle cells.

Fate of cells in the larval regions

To determine the fate of cells in the larval regions of the leg I fed the BUdR diet to the larvae from hatching until the beginning of the final larval instar, at which time they were switched to diet lacking BUdR (see Materials and Methods for details). I predicted that cells that did not divide (or did so only a few times) after this time point would retain their label through the late prepupal and pupal stages, whereas those that underwent rapid proliferation (i.e., those in the primordia) would "dilute" out their labeled DNA so that the BUdR label would eventually become undetectable in these cells. Hence, the presence of BUdR label in cells at these later stages would be used to indicate that they were from the "larval" regions of the leg.

At the beginning of the last larval instar, prior to the proliferation in the adult primordia, almost all the epidermal cells in the leg showed high levels of BUdR signal (Fig. 2.3a, b, d). In most cases, the giant bristle cells were the only cells in the epidermis missing the label. Since these cells must undergo extensive endoreplication of their DNA as they grow during larval life, it is not clear why they seemed to label so poorly. As seen in Fig. 2.3b and 3d, epidermal nuclei in the presumptive primordium regions were as well labeled as those in the surrounding larval regions.

By W+1, the adult leg primordia were well developed and the BUdR label had been lost from these regions (Fig. 2.3c, e). Interestingly, the proximal layer of the tibial primordium appeared to have become a peripodial membrane with large, labeled non-dividing cells (Fig. 2.3e, arrowhead) whereas the distal layer was thickened and had lost

its BUdR labeling (Fig. 2.3e, asterisk). At W+1 the cells outside of the primordium were still strongly labeled (Fig. 2.3c, e).

In the early W+2 leg, the regions of the leg that derived from the adult primordia were conspicuously lacking the BUdR label (Fig. 2.4a), but the larval regions retained a strong BUdR signal. Labeled cells were clustered in the coxa/body wall, ventral femur, ventral tibia and dorsal tarsus (Fig. 2.4a, b). As metamorphosis progressed, the regions of BUdR positive cells diminished both in proportion and actual size. However, even in the mid W+3 leg, these BUdR positive cells were still present in distinct patches (Fig. 2.4c-e). In the femur, a clear band of BUdR positive nuclei remained on the ventral side near the distal boundary of the putative adult femur (Fig. 2.4c, d). A patch in the tibia also formed a band that extended dorsally. It was located on the ventral side just proximal to the base of the distal spurs (Fig. 2.4c, e). The BUdR positive region on the dorsal tarsus was also still present, although it was now confined to a narrow strip near the tip of the leg. Upon closer examination of the morphology of the BUdR positive nuclei in these legs, an increasing proportion was found to be pyknotic (not shown), suggesting that these cells were going through apoptosis.

Around P+6, the epidermal cells are completing their differentiative divisions to make scales and are preparing to secrete their epicuticle (Fig. 2.5a; Jindra et al. 1997). Therefore, if the BUdR labeled cells were found in the leg at this stage, they would most likely persist into the adult stage. Observation of the leg at P+6 revealed that the patch of BUdR labeled cells still remained in the ventral distal area of the femur (Fig. 2.5b, c) and, in the tibia, a well-defined streak of labeled cells was found in the position similar to the mid W+3 leg (Fig. 2.5d, e; compare to Fig. 2.4c, e). I did not observe labeled cells in the dorsal distal tarsus.

Pattern of cell proliferation during the wandering stage

The area of the BUdR-labeled larval regions diminished considerably during the wandering stage (Fig. 2.4). This reduction could be due to cell death in these regions or

alternatively some cells might start dividing in these regions after wandering, thereby, losing their BUdR label. To determine the cause of this loss, I first characterized the pattern of cell proliferation in the leg during the wandering stage by injecting BUdR at different times and looking at its incorporation 6 hours later.

The early W+2 leg epidermis, which had just apolysed from the larval cuticle, had many BUdR positive nuclei in the regions of the adult primordia (Fig. 2.6a). Many cells in the body wall also showed BUdR incorporation (Fig. 2.6b). The larval regions, on the other hand, were distinctly missing BUdR incorporation, indicating an absence of cell proliferation in these areas (Fig. 2.6a, b). Sharp boundaries were observed between these two regions. Counterstaining with propidium iodide showed that all the cells in the larval regions had larger nuclei compared to the BUdR-positive regions (Fig. 2.7g, h), a characteristic of mostly polyploid larval epidermal cells (Kato et al. 1987). The size of the BUdR-negative areas decreased through W+2, however, and the boundaries between the two areas became less sharp by late W+2 (Fig. 2.6c), suggesting that there might be some intercalation between the two cell populations.

By mid W+3, the larval regions occupied only small areas of the leg (Fig. 2.6d). Consequently, BUdR positive-cells were found throughout most of the leg. At this stage some areas that were previously showing a strong BUdR signal had reduced incorporation (not shown). When the larval regions were examined, only few samples showed a BUdR-negative area with a well-defined border (Fig. 2.6e-j). However, in most samples PI counterstain showed small patches of large epidermal nuclei in the larval regions. These nuclei were all free of BUdR incorporation, indicating that at least these patches of cells had not gone through cell proliferation up to this stage (not shown).

Apoptosis occurs exclusively in the larval regions during the wandering stage

The decrease in size of the larval regions might also occur by the removal of the cells by programmed cell death during the wandering stage. The observation of

pyknotic nuclei in the larval regions in the BUdR labeling experiment also suggested this possibility. Indeed, acridine orange (AO) staining showed that apoptosis is prevalent in the larval regions of the early W+2 leg (Fig. 2.7a). In the larval region of the femur, strong AO staining was observed throughout. In the tibial larval region, apoptosis was limited to the proximal and the lateral areas, and the distal lobular region showed only few AO positive cells. Cells in parts of the body wall also showed AO staining. All the AO positive cells were epidermal. In the late W+2 leg, the area of AO positive cells expanded medially (i.e. toward the ventral side) in the tibial region as the tibial lobe diminished in size (Fig. 2.7b). The larval region located in the dorsal tarsus also showed strong AO staining at this time. In the early and the mid W+3 leg, AO signals in the larval regions of the femur and tibia became weaker, while the tarsus still showed a strong AO signal. Throughout the wandering stage, cell death was conspicuously absent in the adult primordia (Fig. 2.7a-d). TUNEL labeling and observation of pyknotic nuclei in propidium iodide stained legs of mid W+2 animals confirmed the results of AO staining (Fig. 2.7e-h).

Discussion

Fates of the larval cell populations in the adult leg

The results of the BUdR labeling experiment and the cell death assays indicate that there are three distinct populations of epidermal cells in the *Manduca* larval leg that differ in their contribution to the adult leg (summarized in Fig. 2.8). The first two populations of cells are polymorphic in that they are part of the larval, pupal, and adult legs. The cells from the adult primordia make the largest contribution to the adult leg. These cells occupy a minor part of the larval leg, but proliferate rapidly at the start of the final larval instar and constitute most of the adult leg epidermis (Figs. 1, 8). Since the adult primordia are located in the specific regions of the larval leg, these cells may

be specified during the embryonic leg patterning. The second population of polymorphic cells resides in the larval regions of the femur and tibia. These cells go through few or no divisions during metamorphosis and contribute to only small parts of the adult leg. Since the boundaries between these cells and those from the primordia were no longer distinct on W+3 (Fig. 2.6d-j), I cannot exclude the possibility that some cells from these regions proliferated during the late wandering stage, therefore losing their BUdR label. Hence, I may have somewhat underestimated their contribution to the adult structure. Except for their stereotyped location, these cells do not appear to differ from the neighboring primordium cells in terms of the types of cuticle that they produce in the adult. The third population of the cells is found in all the larval regions, interspersed with the second polymorphic cell population in the femur and tibia. They produce the larval cuticle during the larval stage and disappear through apoptosis during the wandering stage.

Role of apoptosis in the adult leg development

The metamorphosing leg of *Manduca* goes through the most radical morphological transformation during the wandering stage. I found that apoptosis is prevalent in all larval regions at this stage. In general, apoptosis achieves two things during metamorphosis. First, as in embryonic development, it removes superfluous cells in order to sculpt organs into appropriate size and shape (Dohrmann and Nijhout 1988; Kodama et al. 1995). Secondly, it eliminates cells that are specialized for larval functions (Taylor and Truman 1974). In the metamorphosis of *Manduca* leg epidermis, apoptosis plays a clear morphogenetic role. As a result of the invagination of the adult primordium in the distal tibia (the putative adult tarsus), the tibial larval region is turned into a ventral lobe (Figs. 2d, 7a). Apoptosis appears to eliminate this unnecessary piece of tissue during the wandering stage to ensure the proper adult leg morphology. The zone of cell death is initially restricted to the proximal and lateral ends of this larval region, then, it spreads into the center, leaving only a small group of cells to survive

through metamorphosis. The bristle cells associated with the distal end of this region were found to persist into W+3 and at least two of them still present in the P+6 leg (not shown). The cells in the larval region of the dorsal tarsus appear to be eliminated entirely by apoptosis. In the femoral larval region, the role of apoptosis is less clear. Topologically, the cells in this area do not appear to interfere with the adult leg construction. Within the region, I also did not find any spatial pattern to the loss that would suggest a possible role of apoptosis.

It is difficult to speculate why some cells in the larval regions survive to the adult stage, since they do not appear to be doing anything different from the surrounding epidermal cells. One possibility is that these cells play a role in the patterning of the adult leg. In the insect leg, different regions along the proximodistal axis are specified by expression of unique combinations of patterning genes. It is possible that the larval regions express a certain combination of these patterning genes and that some cells from these regions need to persist in order to provide positional information for the adult leg patterning on the ventral side.

Segmental homology between the larval and adult legs

Morphological observation and surgical experiments have been used to establish the regional correspondence between the larval and the adult legs in several lepidopteran species (Kim 1959; Bodenstein 1949; Miles and Booker 1993; Birket-Smith 1984). In *Manduca*, Miles and Booker (1993) used a homeotic mutant that has an extra pair of thoracic legs on the first abdominal segment. The extra legs in the mutant larvae varied in the number of leg segments present. They showed that the number of segments present in the homeotic larval leg was positively correlated with the number found in the adult leg. Except for the simple morphological observations, none of these studies looked at a normal developing leg. In my BUdR labeling experiment, by noting the positions of the labeled cells at different time points during the adult leg morphogenesis, I was able to directly determine the correspondence

between certain regions in the adult and the larval legs. Observation of adult leg morphogenesis using confocal microscopy also facilitated my analysis. Cells in the ventral larval regions of the femur and tibia end up in the distal area of the respective segments in the adult (Figs. 4, 5, 8). From this it can be inferred that the adult primordia near the distal end of the ventral femur and the ventral tibia of the larval leg mostly give rise to the ventral tibia and tarsus of the adult respectively. The proximal ventral femur of the adult appears to come from the primordium in the ventral larval trochanter. My conclusions are very similar to those of Bodenstein (1949), but differ from Miles and Booker (1993) and Kim (1959), which concluded that the adult primordium in the trochanter does not contribute to the adult femur. Nevertheless, all of the studies including this study agree that the adult primordia in the other segments give rise to the same and more distal segments in the adult leg.

Adult leg development in other holometabolous insects

Three distinct populations of the epidermal cells and their spatial distribution in the larval leg of *Manduca* probably cannot be generalized for all holometabolous insects with biphasic leg development. A histological study of the metamorphosing leg in the beetle *Tenebrio molitor* reports an absence of adult primordium-like tissue (Huet and Lenoir-Rousseaux 1976). Although these authors did not investigate the occurrence of cell death, they suggest that the entire larval leg epidermis contributes to the adult leg (similar conclusions were reported in two other beetle species *Leptinotarsa decemlineata* and *Timarcha violaceonigra*; references in Huet and Lenoir-Rousseaux 1976). Therefore, most of the cells in the larval leg of these beetles are probably polymorphic and they all contribute to the adult leg more or less equally. The manner of larval cell contribution to the adult leg in *Tenebrio* most likely represents a basal state for Holometabola and *Manduca* leg development is more derived.

The most derived mode of leg development in holometabolous insects is observed in those species whose larvae lack the thoracic legs. The legless larval stage has evolved

multiple times in Holometabola (some Coleoptera and Lepidoptera, all Diptera, the Siphonaptera and the higher Hymenoptera). In some species such as *Drosophila* and higher hymenopterans (Cohen 1993; Wheeler and Nijhout 1981), the cells of the adult leg are set aside during embryogenesis or very early in the larval stage and the leg grows as an imaginal disc inside the larval body wall. In what is probably a more ancestral mode of leg development in Diptera, parts of the thoracic epidermis invaginate during the final larval instar and grow as leg discs (Weismann 1866; Lakes-Harlan et al. 1991). The latter case is highly reminiscent of the adult leg primordium in *Manduca* and it is tempting to speculate that these patches of thoracic epidermis are evolutionary derivatives of the adult primordium. Unfortunately, it cannot be said for sure whether imaginal disc development in any of the above species evolved via the *Manduca* mode of development or directly from the more ancestral mode observed in *Tenebrio.* In order to answer this question, adult leg morphogenesis needs to be studied in additional phylogenetically informative groups with biphasic leg development such as mecopterans and lower hymenopterans.

Acknowledgments

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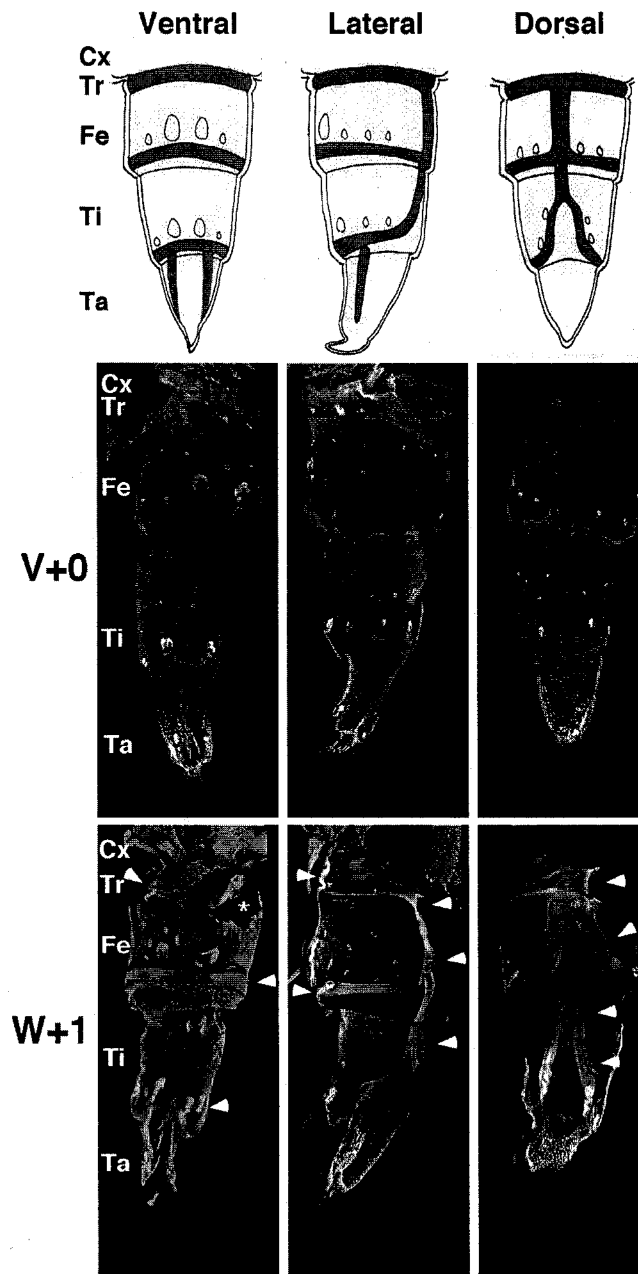

Figure 2.1. Confocal montages showing the adult leg primordia in the larval leg. Legs were split into the ventral, lateral and dorsal halves to expose the leg epidermis beneath the larval leg cuticle. Top panel, schematic drawings of the V+0 leg. Areas shown in gray represent the adult primordium. Circles show the location of some of the giant bristle cells associated with the area outside the primordia (the larval regions). Middle and bottom panels, ventral, lateral, and dorsal views of legs from early (V+0) and late (W+1) in the last larval stage, before the cells of the adult primordia start dividing and after the primordia are well formed, respectively. Legs were stained with propidium iodide to visualize the nuclei of the epidermis. Large nuclei belong to the bristle cells. Arrowheads, proliferating adult primordia. Asterisk, tissue tear. Cx, coxa; Tr, trochanter; Fe, femur; Ti, tibia; Ta, tarsus.

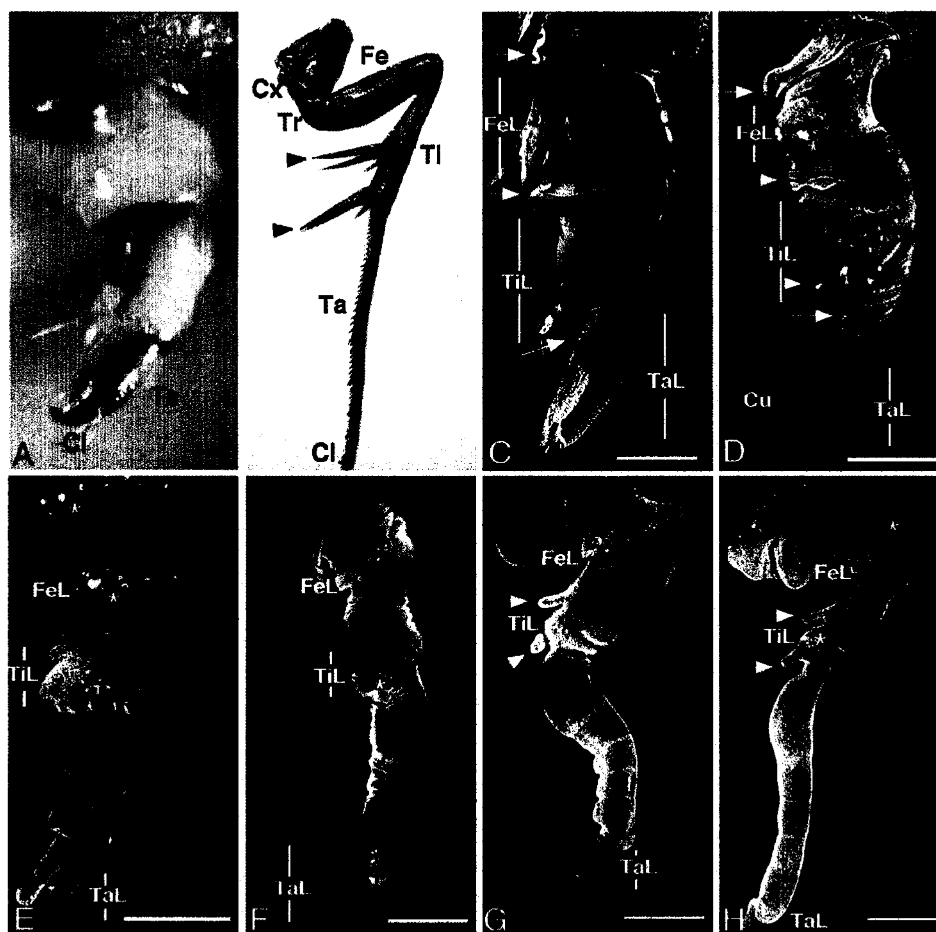

Figure 2.2. Morphogenesis of the adult leg. All of the images are lateral views with the ventral side to the left. (A) V+0 larval leg. (B) Adult leg (metathoracic, T3). Two pairs of spurs are present on the tibia (arrowheads). The leg is approximately seven times longer than the V+0 leg. (C-H) Propidium iodide stained legs at various times after the start of wandering. (C) W+1. The leg epidermis still attached to the larval cuticle viewed from the inside. (D-H) External view of the leg epidermis. (D) Early W+2. Arrows mark the adult primordia. Giant bristle cells associated with the larval regions are also visible. The tibial larval region appears as a lobe hanging over the proximal part of the tibial primordium (arrowhead). (E) Mid W+2. The lobular part of the tibial larval region has disappeared. (F) Late W+2. The folded regions of leg are starting to extend. (G) Early W+3. Tibial spurs are now visible (arrowheads). Proximal portion of the leg are starting to extend. (H) Mid W+3. Tarsal segments can be seen in (G) and (H). Asterisks, examples of bristle nuclei. Cl, claw; Cu, larval cuticle; FeL, femoral larval region; TiL, tibial larval region; TaL, tarsal larval region. For other abbreviations, see Fig. 2.1. Bars, 500 μm.

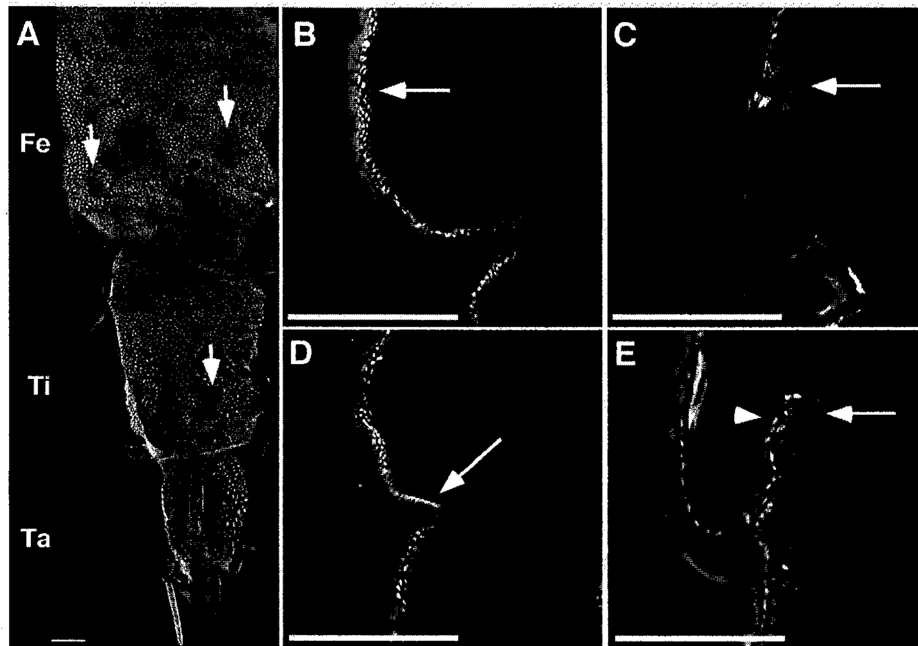

Figure 2.3. BUdR labeled areas in V+0 and W+1 legs of the larvae that have been labeled with BUdR during the first 4 larval stages. (A, B, D) V+0 legs within 2 hr of the last larval ecdysis. (C, E) W+1 legs. (A) All the epidermal nuclei except those of the giant bristle cells (arrows) show a strong BUdR signal. (B, D) Single confocal sections of the area around the adult primordia (arrows) in the femur (B) and the tibia (D). (C, E) Growing adult primordia (arrows) in the femur (C) and tibia (E). In the tibia (E), the cells that have lost the nuclear label, and thus presumably had divided rapidly, appeared to be restricted to the distal layer of the invaginating primordium (asterisk). The cells on the proximal layer retained the BUdR label (arrowhead). Bars, 250 μm. For abbreviations, see Fig. 2.1.

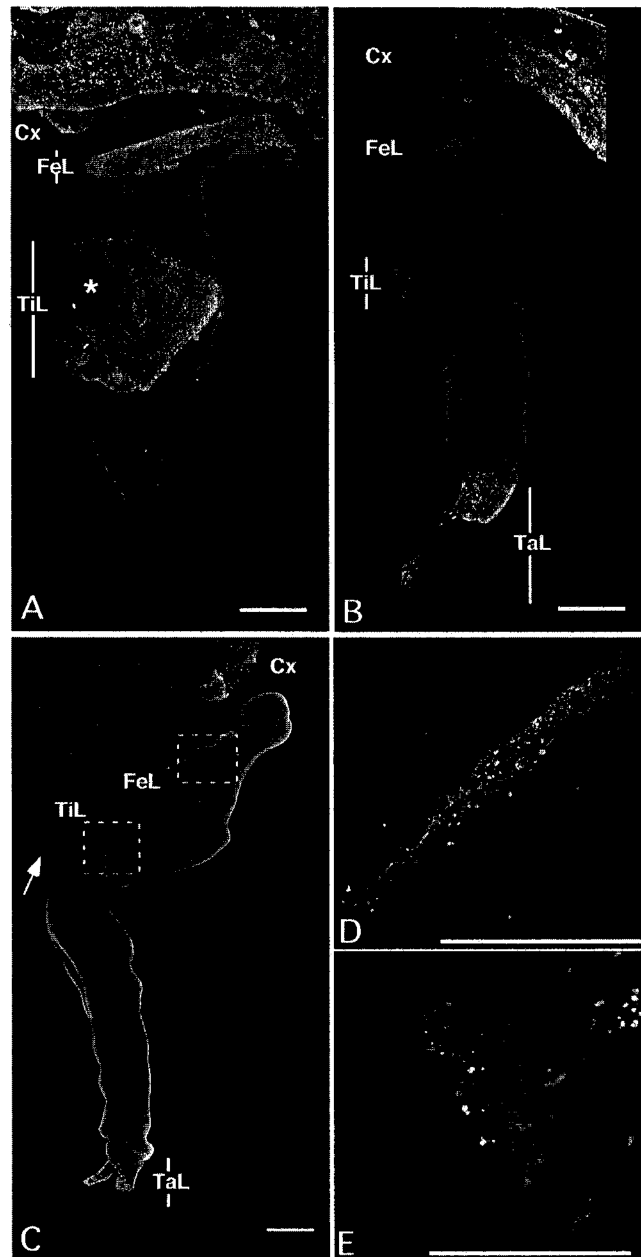

Figure 2.4. Confocal montages showing the BUdR labeled areas in W+2 and W +3 legs from larvae that had been labeled through early larval life (lateral view with the ventral side to the left). (A) Early W +2. Note that the tip of the leg is broken off. (B) Late W +2. (C-E) Mid W+3 T3 leg. There is a small patch of BUdR positive cells on the dorsal side of the tarsal larval region (TaL). Arrow, distal tibial spur. Magnified views of the boxed areas in are shown in (D) and (E). (D) Magnified view of the femoral larval region (FeL). (E) Magnified view of the tibial larval region (TiL). T3, metathorax. Asterisk, a tissue tear. For other abbreviations, see Fig. 2.1. Bars, 250 μm.

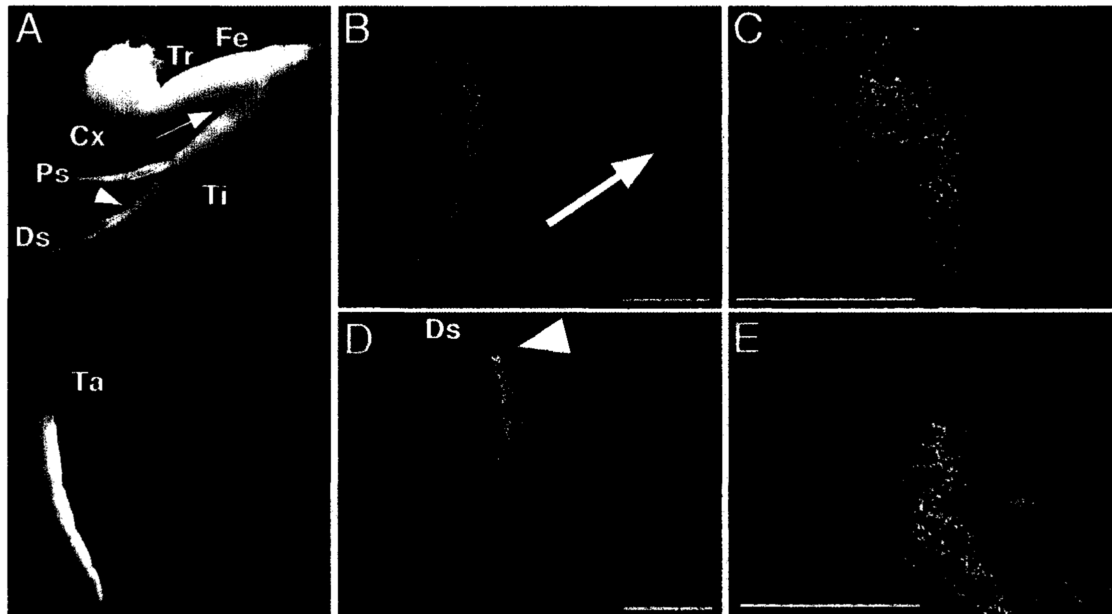

Figure 2.5. BUdR labeled areas in P+6 legs (T3) from animals that had been labeled with BUdR during the first 4 larval stages. (A) Bright field image of the metathoracic leg. The regions corresponding to the femoral and the tibial larval regions are marked by the arrow and arrowhead respectively. (B-E) Confocal images of the area around the femoral (B, C) and the tibial (D, E) larval regions. Red, propidium iodide-stained nuclei; Green, BUdR-labeled nuclei. (B) Arrow points to the femur/tibia border as in (A). (C) Magnified view of the same region, but from another specimen. (D). Arrowhead points to the base of the distal tibial spurs as in (A). (E) Higher magnification view of (D). Ps, proximal tibial spur; Ds, distal tibial spur. Bars, 250 μm.

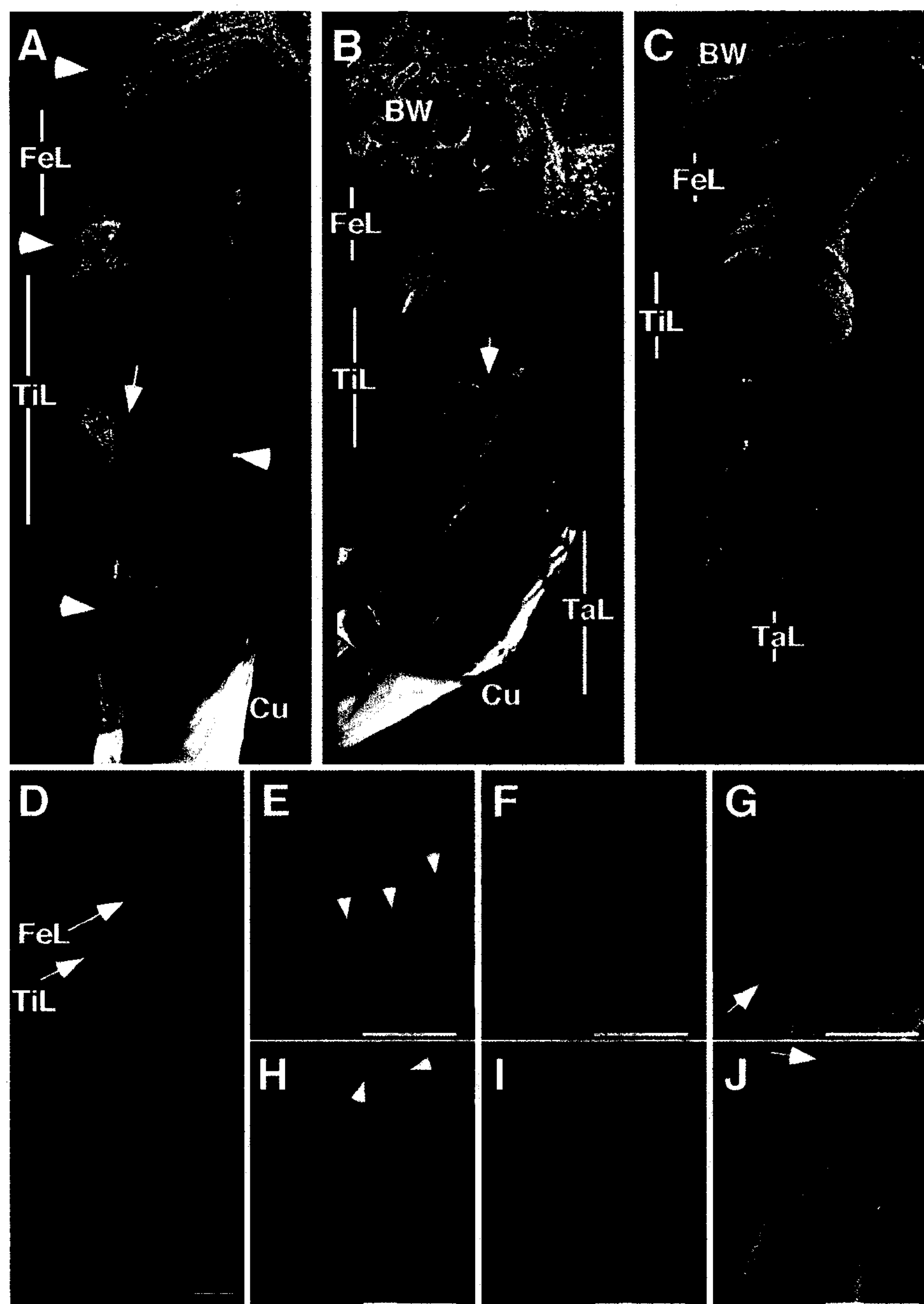

Figure 2.6. Distribution of BUdR incorporation in the legs of the animals injected with BUdR during the wandering stage. (A) Early W+2. BUdR labeled nuclei are only visible in the regions derived from the adult primordia (arrowheads). The larval regions are completely missing the label (FeL, TiL). (B) Mid W+2. (C) Late W+2. The tarsal larval region is also lacking BUdR labeling. Cu, the larval leg cuticle; BW, body wall. In (A) and (B), the signal in the area indicated by the arrow is from the adult primordium behind the fold of the larval region. (D-J) Mid W+3. (D) Propidium iodide stained leg (T3) to show the overall morphology. (E-G) Magnified view of the femoral larval region (FeL) in (D). (H-J). Magnified view of the tibial larval region (TiL) in (D). Red, propidium iodide-stained nuclei; Green, BUdR labeled nuclei. (G) Small area that corresponds to the femoral larval region (arrow) does not incorporate BUdR. (J) In the tibial larval area (arrow) there is also a patch of BUdR free nuclei. Arrowhead, giant bristle nuclei. Bars, 250 μm.

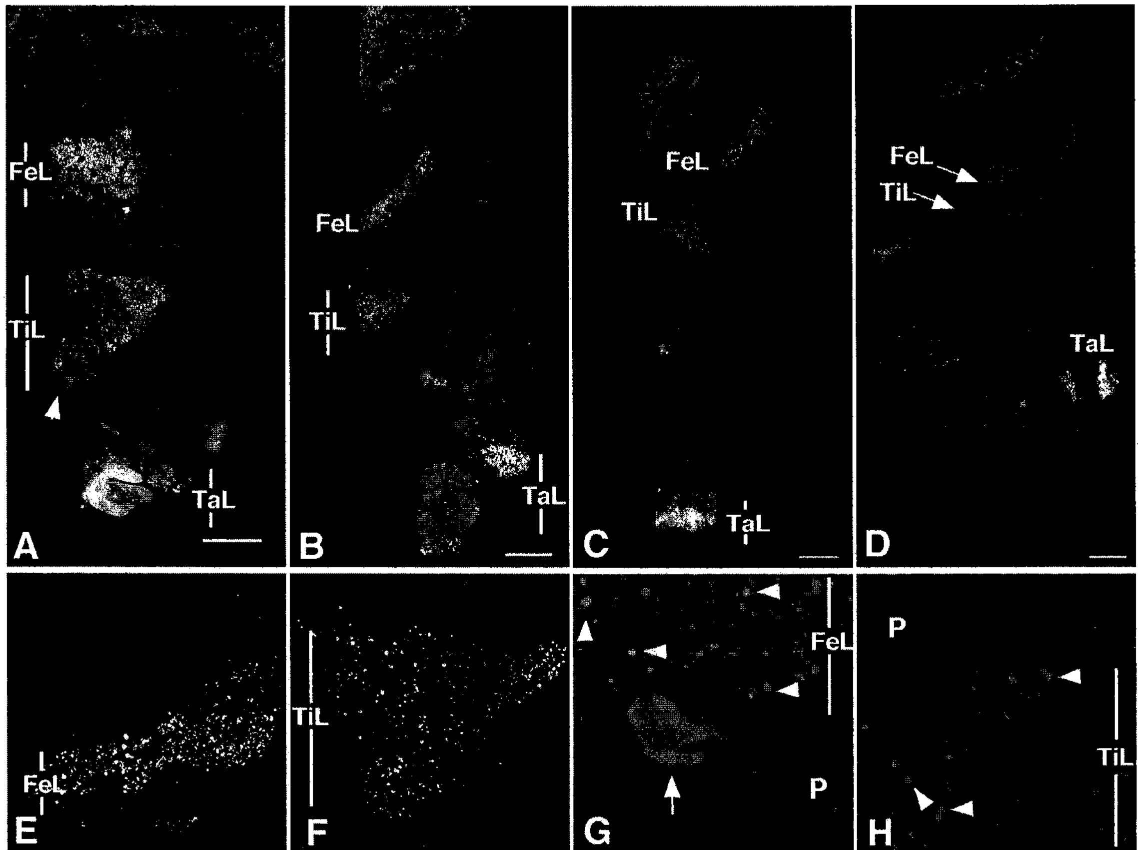

Figure 2.7. Programmed cell death in the leg during the larval-pupal transformation. (A-D) Wandering legs stained with acridine orange (AO) (lateral view). Appearance of these legs looks different from the other figures, because these legs were not fixed. (A) Early W+2. Arrowhead, the tibial lobe. (B) Late W+2. The tarsal larval region (TaL) shows strong AO signal, while the other two larval regions show fewer AO positive nuclei. (C) Early W+3. (D) Mid W+3. (E, F) TUNEL signals from mid W+2 leg. Positive nuclei were restricted to the larval regions. (**e**) Femoral larval region. (**f**) Tibial larval region. (G, H). Propidium-iodide stained mid W+2 leg to show pyknotic nuclei. The images show the boundaries between the adult primordium (with smaller nuclei) and the femoral larval region (G), and between the primordium and the tibal larval region (H). Highly condensed pyknotic nuclei (arrowheads) are only visible in the larval regions. FeL, femoral larval region; TiL, tibial larval region; TaL, tarsal larval region; P, adult primordium. Arrows, giant bristle nuclei associated with the larval regions. Bars, 250 μm.

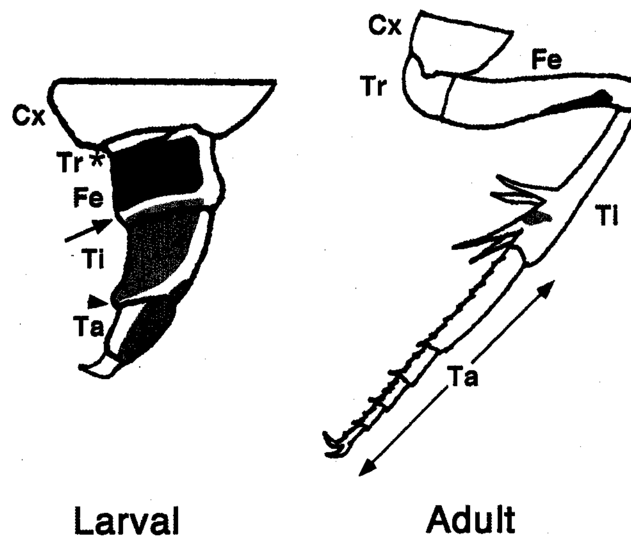

Figure 2.8. Fate of the larval regions in the adult leg development. Metathoracic leg (T3) is shown. White, adult primordia. Asterisk, ventral primordium in the trochanter; Arrow, ventral primordium in the distal femur; arrowhead, ventral primordium in the distal tibia; Red, femoral larval region. Blue, tibial larval region. Green, tarsal larval region. Colors in the adult leg indicate areas where the persistent cells from the colored regions in the larval leg are found. The tarsal larval region does not appear to contribute to the adult leg. The relationship between the epidermal cells in the larval and the adult coxa (pink) were not addressed in this study.

Chapter 3

Molecular patterning mechanism underlying biphasic leg development in *Manduca sexta*

Introduction

Holometabolous insects comprise one of the most speciose groups of animals on our planet. Their success is largely due to their ability to produce disparate larval and adult forms highly adapted to utilize distinct ecological niches. Larval forms are produced during embryogenesis and the distinctly different adult emerges following metamorphosis. During the past few decades, molecular mechanisms underlying patterning of various organs in insects have been studied extensively in *Drosophila* and to a lesser degree in a few other species. However, these studies rarely focused on organs that undergo radical morphological changes to function in both the larva and the adult (Friedrich and Benzer 2000; Lieu and Friedrich 2004; *Tribolium* embryonic legs – e.g. Prpic et al. 2001). Therefore, how molecular patterning mechanisms are utilized by holometabolous insects to produce stage-specific organ forms remains unknown.

In order to elucidate the molecular patterning system underlying the generation of stage-specific organ forms, I have investigated the development of the thoracic leg in the tobacco hornworm *Manduca sexta.* The larval leg of *Manduca* is produced during embryogenesis (Fig. 3.1A, C). This simple leg consists of the five primary segments (coxa, trochanter, femur, tibia and tarsus with a pretarsal claw) characteristic of all insect legs, but it is only capable of grasping motions (Kent et al. 1996). Metamorphosis of the leg, which begins during the last larval instar, transforms this structure into a form typical of adult insects (Fig. 3.1B; Tanaka and Truman 2005). This leg is 7 times greater in length relative to the size in the final instar larva, with each segment undergoing differential growth. Most prominently, the tarsal segment, which consisted of one segment in the larva, is now divided into 5 subsegments (Fig. 3.1B). Additional structures such as spurs develop on the tibia (Fig. 3.1B). Since the most

radical difference between the two legs in *Manduca* is along the proximal-distal axis (PD axis), I have examined the patterning of this axis.

The molecular mechanism of PD axis patterning is well understood in the leg imaginal disc of *Drosophila* (Kojima 2004). Shortly after allocation of the leg imaginal discs during embryogenesis, a secreted signaling molecule of the TGF-beta family, Decapentaplegic (Dpp), is produced in a dorsal stripe and another signaling molecule of WNT family, Wingless (Wg), in a ventral stripe just anterior to the anterior-posterior compartment boundary. The cells in the presumptive tip of the leg receive high concentration of the two signals and activate the homeobox gene *Distal-less* (*Dll*), which specifies these cells to be the distal region of the adult leg (Fig. 3.2A top; Cohen et al. 1989). In the future proximal region of the leg, where the levels of Dpp and Wg are low, *homothorax* (*hth*) and *teashirt* (*tsh*) are expressed (Fasano et al. 1991; Abu-Shaar and Mann 1998; Wu and Cohen 1999). Hth binds to the ubiquitously expressed homeodomain protein, Extradenticle (Exd), and transports it to nucleus, where the three molecules act to specify the proximal fate (Rieckoff et al. 1997). In the second instar, *dachshund* (*dac*), a gene encoding a novel nuclear protein, is activated in the intermediate region of the disc by medium levels of Dpp and Wg (Fig. 3A top; Mardon et al. 1994). At this stage, there is a mutual repression between *hth* and *dac*, *tsh* and *dac* and *Dll* and *dac*, which creates non-overlapping expression domains of these proteins along the PD axis (Fig. 3.2A top; Dong et al. 2001; Kojima 2004).

Beginning in the late 2nd instar, a set of genes that subdivides the tarsal segment starts to be expressed within the *Dll* domain. The first to be expressed is *spineless* (*ss*), a homolog of the mammalian dioxin-receptor, which is required for proper development of the second to fourth tarsal segments (Ta2-Ta4) (Duncan et al. 1998). In the early 3rd instar, *ss* expression disappears and is replaced by expression of a BTB domain-encoding genes, *bric-a-brac* 1 and 2 (hereafter referred to as *bab*), which are also required for Ta2-Ta4 development (Fig. 3.2A middle; Godt et al. 1993; Couderc et al. 2002). At this stage, *dac* represses *bab* expression setting the proximal border of the *bab* domain (Chu et al. 2002). At the distal-most region, *aristaless* (*al*) is activated to specify the pretarsal claw (Fig. 3.2 middle; Campbell et al. 1993). After the early 3rd

instar, regulation of PD genes becomes independent of Wg and Dpp signaling. Removal of the functions of the two signaling pathways does not affect the PD patterning of the leg at this point, because the PD genes that have been activated regulate themselves and other genes downstream. In addition, the EGFR signaling starts to regulate the patterning in the distal-most region (Galindo et al. 2002; Campbell 2002). In the late 3rd instar, the final expression patterns of the PD genes are established (Fig. 3.2A bottom). There are now substantial overlaps between the expression domains of the PD genes. *bab* and *dac* are co-expressed in the presumptive Ta1-3, while *Dll* and *dac* overlap in the distal tibia and proximal tarsus (Chu et al. 2002). *hth*/*tsh*/nuclear Exd, *Dll* and *dac* share expression domains in the distal trochanter and the proximal femur. *al* and *bab* are now also expressed in the proximal domains. Finally, *bab* expression domain resolves into four stripes of strong expression in the distal regions of Ta1-Ta4 flanked by areas of lower expression (Godt et al. 1993; Couderc et al. 2002; Chu et al. 2002). This modulation of *bab* expression is thought to be necessary for proper formation of the tarsal joints (Chu et al. 2002).

Outside of *Drosophila*, the expression patterns of some of these genes have been studied in developing embryonic legs in several orders of hemimetabolous and holometabolous insects that have more ancestral modes of leg development (see Angelini and Kaufman 2005 for a comprehensive review). These include crickets, a grasshopper, a bug, a beetle and some lepidopterans. In all species examined, the early PD genes, *Dll*, *dac*, *hth* and nuclear Exd are expressed in a similar pattern suggesting conservation of their function (expression patterns for crickets shown in Fig. 3.2). Results from functional studies using RNAi in *Oncopeltus* (Angelini and Kaufman 2004) and a mutant strain in *Tribolium* (Beermann et al. 2001) indicated that the genes specify similar sets of segments as in the fly. *al* in *Tribolium* and *Gryllus*, a late PD gene, was expressed in domains similar to *Drosophila* and a RNAi knockdown study in the former species resulted in loss of the claw (Miyawaki et al. 2002; Beermann and Schroder 2004). Another late gene, *bab*, is expressed in a similar spatial and temporal pattern in developing cricket leg (Erezyilmaz et al. 2004). *wg* is also expressed in a similar manner in all species examined (Jockusch et al. 2000; Niwa et al. 2000; Nagy

and Carroll 1994; Angelini and Kaufman 2005), although RNAi knockdown experiments suggested that it is not required for PD axis patterning outside of the Holometabola (Miyawaki et al. 2004; Angelini and Kaufman 2005; Ober and Jockusch 2006). *dpp* expression patterns in other insects are more dynamic and differ from *Drosophila* (Jockusch et al. 2000; Niwa et al. 2000; Angelini and Kaufman 2005; Yamamoto et al. 2003). However, outside *Drosophila*, *dpp* has yet to be shown to be necessary for PD patterning in the leg (Ober and Jockusch 2006).

Drosophila shows a highly derived mode of leg development as their larva have secondarily lost their thoracic legs. The adult leg grows as an imaginal disc, an invagination inside the larval body wall, which everts during the prepupal period. The early phase of the patterning process takes place during embryogenesis when the presumptive leg disc is divided into proximal and distal halves. It is not until after the 2nd instar that most of the patterning takes place (Fig. 3.2). Based on data from the two orthopterans and *Oncopeltus*, hemimetabolous insects appear to complete the entire patterning process during embryogenesis (Fig. 3.2B), giving rise to the adult-like leg of the hatchling. In less derived holometabolous insects such as *Manduca*, I hypothesized that this patterning cascade is initiated during embryogenesis, but is interrupted before it reaches the final adult pattern. The arrested expression patterns of the PD genes are then used to produce the simple larval legs. During metamorphosis, the patterning process is resumed to achieve the mature expression needed to pattern the adult leg. I have tested this hypothesis by examining the expression patterns of *Dll*, *dac*, *exd*, *bab* and *al* during the larval and the adult leg development. My results were consistent with the idea that the arrested pattern of the PD gene expression produces the larval leg. In addition, I find that during the remodeling of the leg, cells change expression of the PD patterning as their segmental position shifts. My results will be discussed in context of the homology between the larval and the adult leg segments as well as the evolution of larval leg form in Holometabola.

Materials and Methods

Experimental animals

Larvae of *Manduca sexta* (L) were raised in individual containers on an artificial diet (Bell and Joachim, 1976) at 26°C under a long-day photoperiod (17hr light and 7 hr dark). After they initiated wandering, larvae were placed in holes bored in wooden blocks for pupation. The animals were chilled on ice before dissection.

The developmental stages of animals were determined with reference to developmental transitions. The day of ecdysis to the last larval instar was designated V+0. A day after V+3, larvae began to wander (W+0). The wandering stage lasted approximately 4 days after which the animals pupated (P+0). For more precise developmental stages, the larvae were staged as the number of hours after ecdysis (e.g. V< 2hrs).

The embryos were allowed to develop at 26°C (17L:7D) and were staged according to Broadie et al. (1991). Ages are given in percentage of overall embryonic development (%E).

Cloning of Msdac and Msdpp

To clone *Manduca sexta dachshund* (*Msdac*), total RNA was extracted from embryos with TriZol (Life Technologies, Grand Island, NY) according to the manufacturer's instruction. cDNA was produced using SuperScript II Reverse Transcriptase (Life Technologies, Carlsbad, CA). Nested degenerate PCR was used to isolate the orthologous genes. *Msdac* was initially isolated in two overlapping fragments. To isolate the 3' fragment, the outer primers *dac-fw-1* and *dac-bw-1*designed for *Tribolium* by Prpic et al. (2001) were used followed by the inner primers *dac-bw-2* (Prpic et al. 2001) and *lep5A* (5'ACNCARTTYCCNGGNCAYCCNTTYAARAA 3'). For the 5' fragment, outer primers used were *dac-fw-1* (Prpic et al. 2001) and *lep3A* (5' TGNCCYTTDATDATNGANCCRTCNGG 3'). The inner primers were *dac-fw-2* (Prpic et al. 2001) and *lep3B* (5'CATNARNGCRTGYTGRTGCATRAANCCCAT3'). An 867 bp fragment spanning the two fragments above was obtained with *Msdac*

specific primers Dac5A (5'GCATCCTGCGCGGTTTGGGCGCCAT 3') and Dac3A (5'GCAGGCCTTGTATATTCCTGAGCAGGG 3'). *Manduca Dll* clone was a generous gift of Dr. Richard Vogt. The sequence for *Msdac* was submitted to Genbank under the accession number DQ985170.

Immunocytochemistry

The eggs were placed in bleach to soften the chorion and dissected in *Manduca* saline (Riddiford et al., 1979). The embryos were fixed in 3.7% formaldehyde in phosphate-buffered saline, pH 7.2 (PBS: 130mM NaCl, 7mM Na_2HPO_4, 3mM NaH_2HPO_4) for 30 min followed by rinses in PBS with 1% Triton-X 100 (PBS-TX; Sigma, St Louis, MO). For the larval stages, the legs were dissected as described previously (Tanaka and Truman 2005; Chapter 2).

The tissues were blocked in 5% donkey serum in PBS-TX for 30 min, followed by a rinse in PBS-TX. Antibody concentrations used were: anti-Bab2 1:1000-2000 (Couderc et al. 2002), anti-Al 1:1000(Campbell et al. 1993), anti-Exd 1:20(Aspland and White 1997) and anti-PH3 1:1000 (Zymed, San Francisco, CA). Secondary antibodies were Alexa 488 (Molecular Probes, Eugene, OR) and TxR (Jackson Immunoresearch Laboratories, West Grove, PA) used at 1:1000. After the final rinse, the tissues were dehydrated through an ethanol series, cleared in xylene, and mounted in DPX (Fluka, Buchs, Switzerland)

in situ hybridization

Digoxigenin (DIG) -probes were made using MaxiScript kit (Ambion, Austin, TX). Probes were hydrolyzed to optimal lengths by alkaline hydrolysis. The optimal durations for hydrolysis of the probes were determined to be 40 min for *dac* and 40 min for *dpp*. Hydrolysis was not necessary for the *Dll* probe. The dissection procedures were similar to above with few modifications. For the embryos, the eggs were placed in 50% bleach, followed by rinses in PBS-TX and distilled water, then fixed for one to 2 hours on a shaker. Embryos were then dissected in PBS and further fixed overnight at 4°C (Kraft and Jackle 1994). For the larval and pupal legs, the initial dissection was

carried out in *Manduca* saline, then fixed for 2 hours. Further dissection was carried out in PBS, followed by an overnight fix at 4°C. Tissues were then rinsed twice in 5% 0.5M EGTA in MeOH and stored at -20°C. The *in situ* procedure was carried out according to the modified protocol by Panganiban et al. (1995), which was based on Tautz and Pfeifle (1989).

For simultaneous detection of protein and mRNA, first the immunocytochemistry was carried out as above using RNAse-free reagents followed by a post-fix for 40 min. The tissues were then taken through an *in situ* hybridization protocol. For fluorescent detection of the DIG probe, the tissues were incubated with mouse anti-DIG antibody (Roche, Indianapolis, IN) followed by horseradish peroxidase conjugated anti-mouse antibody in Tyramide Signal Amplification Kit (Molecular Probes, Eugene, OR). The rest of the fluorescent detection was carried out according to the manufacturer's manual and Kosman et al (2004).

For *in situ* hybridization on sections, tissues were fixed overnight, then cryoprotected with a sucrose series (10%, 20%, 30% in PBS), embedded and frozen in OCT embedding medium (Sakura, Torrance, CA). Sections were cut on a Leica CM1850 cryostat (Leica, Wetzlar, Germany) and stored at -80°C. For hybridization, the sections were incubated in 2μg/ml proteinase K in PBT for 15 min at 37°C, followed by a wash in 0.2% glycine in PBT. The tissues were post-fixed for 20 min, rinsed in PBT and soaked in 1M triethanolamine (pH 8.0) for 2 min. Acetic anhydride was added to the final concentration of 2.4 μl/ml and the tissues were incubated for 10 min on a slow shaker. The slides were rinsed in 2XSSC and pre-hybridized in the hybridization buffer for 1 hr at 37°C. Probes were added to slides, covered with a wide coverslip and incubated in a humid box at 55°C for 24 hrs. Slides were washed in the hybridization buffer, 2XSSC, 1XSSC and 0.1XSSC for 1 hr at 55°C respectively. They were equilibrated in RNAse buffer (0.5M NaCl, 1mM Tris, ph 8.0, 1mM EDTA, ph 8.0) and incubated with 20 μg/ml RNAse A for 30 min at 37°C. After a rinse in PBT, the slides were blocked as above for 2 hrs at RT, then incubated with 1:1500 AP anti-DIG antibody under a coverslip for 2 hrs at RT or overnight at 4°C. The slides were washed

4 times in PBT over an hour, followed by the color reaction (Panganiban et al.1995) under a coverslip.

Image collection and analysis

All fluorescent images were collected using confocal microscopy and processed with the NIH Image program (http://rsb.info.nih.gov/nih-image) and Adobe Photoshop (Adobe Systems Inc., San Jose, CA). To make Z-series projections of the postembryonic legs, non-epidermal areas were removed from each confocal section and then projected. The images for *in situ* hybridization were collected on a light microscope. For the larval leg, images from several focal planes were projected using Combine Z 5.2 (http://www.hadleyweb.pwp.blueyonder.co.uk). To produce images of the whole leg, the individual pieces were assembled into a montage using Photoshop. Most of the legs and all the legs older than mid W+2 were from the metathorax (T3) segment. The T3 leg has two pairs of spurs on the tibia, which were convenient positional markers. The spurs became visible around mid W+2.

Results

Manduca leg development

During *Manduca* embryogenesis, the thoracic leg buds forms around 15% E (Broadie et al. 1991). Morphological segmentation occurs between 18% and 36% E except for the trochanter (Fig. 3.1C). Segmentation between the presumptive coxa and the more distal segments and between tibia and tarsus appears first, followed by a demarcation between the femur and the tibia. The segmentation between the tibia and tarsus becomes inconspicuous until later embryonic stages. The trochanter does not appear as a distinct segment until very late in embryonic development. Morphogenesis of the claw begins by 55% E. Since most of the segmentation of the leg is complete by 36% E, stages later than 48% E were not included in this study.

All the epidermal cells in the leg make the larval leg cuticle during the subsequent larval stages. However, early in the last larval instar (~ 24 hr after ecdysis), the "adult leg primordia" detach from the cuticle and begin rapid proliferation. The adult leg primordia are a subset of the epidermal cells located in specific locations in the larval leg (Fig. 3.1D; colored area). These cells eventually give rise to most of the adult leg (Tanaka and Truman 2005). Areas outside the adult leg primordia are referred to as the larval regions (Fig. 3.1D; grey area). The larval regions go through extensive cell death and only contribute to minor parts of the adult leg epidermis (Fig. 3.1D; Tanaka and Truman 2005).

Morphogenesis of the adult leg takes place through the wandering (W) and prepupal stages and by W+3 the leg has attained an adult-like morphology (Fig. 3.1D, B). In the early W+2 leg, the segmentation in the tarsus is visible (Fig. 3.1, arrowheads) and the tibial larval region appears as a lobe hanging over the ventral side of the tarsal primordium (Fig. 3.1. arrow). In the mid W+2 leg, the proportion of the larval regions relative to the whole leg decreases significantly and the folded part of the tarsus has extended. By early W+3, the adult leg morphology is apparent including two pairs of the tibial spurs (arrowheads). Based on the morphological observations of the developing wandering leg and the location of the surviving larval cells in the adult leg, I concluded that the primordium in the larval trochanter gives rise to the adult femur (and probably the trochanter), the one in the larval femur to the adult tibia and the one in the larval tibia to the distal-most part of the adult tibia and the adult tarsus (Fig. 3.1D; Tanaka and Truman 2005).

Expression of PD patterning genes during the larval leg development

Dll mRNA expression was observed in the presumptive leg bud (before the limb outgrowth was visible) as early as 12% E (not shown). At 18% E, its expression domain was in most of the growing limb bud except the most proximal part (Fig. 3.3A). By 24% E, this expression domain resolved into the "sock and ring" pattern with the

sock extending distally from the femur-tibial boundary and the ring in the presumptive trochanter region (not shown). This pattern persisted through 36% E and beyond (Fig. 3.3E; not shown). These results are consistent with the Dll protein expression patterns published previously for *Manduca* (Zheng and Booker 1999).

At 24% E, nuclear Exd was detected in the body wall and the presumptive coxa (Fig. 3.3D). By 36% E the distal limit of the expression domain was found to be within the presumptive trochanter (Fig. 3.3H). At this stage, the intensity of immunoreactivity in the trochanter was lower compared to the more proximal part of the Exd expression domain (Fig. 3.3H). Cytoplasmic expression in more distal areas has been reported in other insects (Angelini and Kaufman 2005), but was not seen in *Manduca* embryo.

dac mRNA was first detected in the leg by 18% E in the intermediate region of the limb bud (Fig. 3.3B). The expression appeared to arise within the early *Dll* expression domain (Fig. 3.3A). This intermediate expression was maintained through 36% E and localized to the femur and tibia and possibly in the trochanter (Fig. 3.3F).

Bab expression appeared in the leg by 24% E as a single band in the presumptive tarsus minus the distal-most part (Fig. 3.3C). This expression pattern was maintained through 36% E (Fig. 3.3G). Since the distal border of the *dac* expression domain and the proximal border of Bab expression domain were around the tibia/tarsus border, presence of overlap between the two domains was examined. Double-fluorescent staining of embryos at 24% E (not shown) and 36% E showed no overlap between the two domains (Fig. 3.3I-K).

Al protein, which is required for claw development, was expressed weakly at the tip of the leg at 24% E (Fig. 3.3L). There was a stronger signal at the lateral base of the leg. This latter domain was not unique to the leg-bearing thoracic segments, but was seen in all the abdominal segments as well (not shown). The embryonic expression patterns of the PD genes are summarized in Fig. 3.11.

Expression of PD patterning genes in the developing pupal leg

In order to investigate the molecular patterning of the adult leg, the expression patterns of the four genes were examined on W+2 and W+3. These developmental stages were chosen because morphogenesis of the pupal leg, which prefigures the morphological features of the adult leg, takes place during this period (Fig. 3.1D).

Dll mRNA was detected by *in situ* hybridization on frozen sections (Fig. 3.4A-C). In the W+2 leg, *Dll* expression was observed in most of the leg, except in the coxa and a ventral patch and a dorsal patch derived from the larval femur (Fig. 3.4A). The ventral patch corresponded to the "larval regions" in the femur, which goes through extensive apoptosis at this stage. On W+3, the expression domain of *Dll* mRNA remained the same (Fig. 3.4B, C) and, at this point, it was possible to assign the expression domains to the adult segments (Fig. 3.4I). *Dll* was expressed in the entire leg distal to the coxa except in the area around the femur-tibia boundary.

During W+2 and W+3, *dac* mRNA was still expressed in the presumptive femur, tibia and trochanter of the pupal leg (Fig. 3.4D, E). Additionally, the expression domain expanded into the proximal tarsus during this period (Fig. 3.4D, E).

The expression pattern of Exd in the developing pupal leg also changed from the embryonic one. In the W+2 leg, the strongest nuclear Exd signal was still in the coxa and part of the trochanter (Fig. 3.4F). However, now, a weak nuclear expression of Exd was observed in most of the femur including the area derived from the larval region in the larval femur. In the W+3 leg, the distal limit of nuclear Exd expression domain was determined to be in the distal femur (Fig. 3.4G-I).

In the developing pupal leg, Bab expression was observed in most of the tarsus and in a new domain in the presumptive trochanter (Fig. 3.5A inset). Expression was missing from the tarsal tip and the proximal part of the presumptive Ta1 (Fig. 3.5A). Within the tarsal expression domain of Bab, four stripes of more intense expression were observed in early W+2 around the time when morphological segmentation of the tarsal segments appeared (Fig. 3.5C). This pattern persisted to late W+2 (Fig. 3.5B). Both the stripes of higher expression and morphological segmentation became less

pronounced in the W+3 leg (not shown). This could be due to the start of the cuticle production, which makes the epidermal cell less accessible to the antibody and which causes wrinkles in the leg. However, the four stripes were observed again in the developing adult tarsus by P+4. At this point, the locations of the rings could be identified as the future joints of the leg. Bab antibody also labeled the scale precursor cells in the P+4 leg (Fig. 3.5D).

Both the distal border of the *dac* expression domain (Fig. 3.4D) and the proximal border of the Bab expression domain (Fig. 3.5A) were located within the proximal tarsus during pupal development. In the developing larval leg, the two expression domains were mutually exclusive. In order to examine whether an area of overlap arose between these two domains at metamorphosis, their expression patterns were determined on alternate sections from single legs. Immunocytochemistry with Bab antibody (Fig. 3.6A) and *in situ* hybridization with *dac* (Fig. 3.6B) on W+2 legs showed that their expression domains now overlapped in the proximal tarsus (Fig. 3.6C). The proximodistal position of the overlap corresponds to the distal part of the first tarsal segment and possibly the proximal part of the second tarsal segment.

Al expression was only examined in the developing adult leg (during the pupal stage), as the distal tip of the wandering leg was too fragile for dissection. In contrast to the weak expression at the tip of the leg in the embryo, Al was expressed at a high level in the dorsal side of the developing adult claw (Fig. 3.7), an expression pattern similar to that observed in the *Drosophila* leg imaginal disc. Al expression at the base of the leg was not examined for this stage. The expression patterns of the PD genes during the pupal leg development are summarized in Fig. 3.11.

The adult primordia change their PD gene expression during the last larval instar

The cells in the adult primordia change their segmental identities during metamorphosis (Fig. 3.1D; Tanaka and Truman 2005; Kim 1959; Miles and Booker 1993). My observation of PD gene expression patterns during the wandering stage

suggested that gene expression might also change in these cells (Fig. 3.11). For example, both the larval and adult tibia express *Dll* (Fig. 3.11). However, the adult tibia is derived from the primordium in the larval femur, which did not express *Dll* during its development. This implied that the primordium in the larval femur turned on *Dll* expression *de novo*.

In order to confirm this observation, expression patterns of *Dll, dac* and Bab were examined in the leg of the last larval instar. In the freshly molted last instar larva (V< 2 hrs), *Dll* mRNA expression was not observed in the leg epidermis including the adult primordia (Fig. 3.8A). In contrast, *dac* mRNA was expressed ubiquitously at a high level in the leg (Fig. 3.8E). However, it was not certain whether this expression reflects the actual level of *dac* mRNA expression or non-specific binding of the probe for two reasons. First, the sense control probe also showed similar ubiquitous expression (data not shown). In addition, our preliminary results from RT-PCR on the last larval instar legs showed that *dac* expression is quite low at this time (not shown). The Bab expression pattern was slightly modulated in the freshly molted last instar larva compared to that during embryogenesis. The tarsus still showed strong expression both on the dorsal and the ventral sides (Fig 3.9. C, F). In addition, the tibia also showed a weak but clear Bab immunoreactivity (Fig. 3.9 B, E). The femur showed very weak to background level signals (Fig. 3.9 A, D).

The expression patterns of these genes changed dramatically in the V+3 leg in which the adult primordia were well developed. One feature that the expression patterns of all three genes shared in common at this developmental stage was that their expression was mostly limited to the primordia (Fig. 3.8, 9 G-J). Little to no expression was detected in the epidermal cells in the larval regions (except Bab. See below). *Dll* mRNA was expressed in all three primordia (in the trochanter, femur and tibia) (Fig. 3.8B-D). However, its expression was missing from the proximal region of the femoral primordium both on the dorsal and the ventral side (Fig. 3.8B, D). *dac* mRNA was also expressed in all the primordia (Fig. 3.8F-H), but in the tibial primordia, its expression was restricted to the proximal half, corresponding to the proximal-most part (ta1) of the pupal tarsus (Fig. 3.8H). In contrast to *Dll* and *dac*, Bab was expressed at a significant

level in larval cells of the tarsus (Fig. 3.9H) as was also seen in V< 2 hrs leg (Fig. 3.9C). Bab expression in the primordia was limited to the one in the tibia (Fig. 3.9G, I, J). Specifically, its expression domain was restricted to the distal part of the primordium in a pattern that was opposite to that seen for *dac* (compare Fig. 3.8H, 3.9I). Bab expression also appeared in the primordium in the ventral trochanter at a moderate level on this day of the last larval instar (not shown).

The expression patterns of these genes in the developing primordia show that many cells in the primordia express new PD genes in accordance with their future segmental positions in the adult leg. *Dll* mRNA is newly expressed in the primordium originating in the larval femur, which did not express the gene during the larval leg development (Fig. 3.3E, 3.8B, C). This primordium later gives rise to the tibia, which expresses *Dll* in the adult legs of all insects. Likewise, Bab is expressed *de novo* in the cells in the adult primordium in the larval tibia.

Timing of changes in the PD gene expression relative to the development of the adult primordium

The first known event in the development of the adult leg primordia is the transcription of the BTB/POZ transcription factor *broad*, which occurs by 12 hrs after ecdysis to the last larval instar (Truman et al. 2006). Proliferation within the primordium, which can be detected by PH3 antibody staining or labeling with BrdU, starts between 24 hrs and 30 hrs. The time course of events in the eye primordium is very similar to those in the leg. In the former, Allee *et al.* (2006) reports that *broad* transcription occurs between 12 and 18 hrs, apolysis around 20 hrs and proliferation around 24 hrs. Based on this observation, the apolysis of the tibial primordium is expected at around 20 hrs. In order to determine when the change in the expression of PD genes occur in the adult primordium relative to these events, *Dll* mRNA expression was examined in the leg during the first 24 hrs of the last instar. I found that the *Dll* transcripts were present in the tibial primordium as early as 18 hrs after ecdysis (Fig.

3.10B, D) and a moderate expansion of the expression domain was observed by 24 hr (Fig. 3.10C, E). To confirm that the transcription of this PD gene occurred before the cell proliferation, the legs from the same larvae were stained for PH3. In both 18 hr and 24 hr legs, no PH3 signal was detected (not shown; Fig. 3.10A). This observation places the transcription of *Dll* as one of the earliest known steps in the development of the adult leg, although it is unknown whether *Dll* transcription occurs before, after or simultaneously with *broad* expression.

Discussion

Molecular patterning of the larval and the adult leg

In this study I have characterized the expression patterns of five PD genes that pattern the PD axis of the simple larval leg and the more elaborate adult leg in *Manduca*. My hypothesis was that the adult patterning process is truncated prematurely during embryogenesis and this truncated PD gene expression pattern is used to make the larval leg. During metamorphosis the patterning process resumes to generate the mature expression pattern of the PD genes, which then gives rise to the adult leg. My results were consistent with this hypothesis. We found that the expression pattern of the PD genes that gave rise to the adult leg of *Manduca* (Fig. 3.11 bottom) was very similar to those of *Drosophila* (Fig. 3.2A; Kojima 2004) and crickets (Fig. 3.2B; Inoue et al. 2002; Miyawaki et al. 2002; Erezyilmaz et al. 2004), indicating that the PD gene expression patterns used to make the adult legs are highly conserved in insects.

Examination of the five PD genes during the larval leg development in *Manduca* revealed that this simple leg was produced by what appeared to be a truncated version of the conserved adult expression pattern (Fig. 3.11 top). In this truncated pattern, Exd, Dll and Dac were already expressed in an adult-like pattern in the presumptive trochanter, femur and tibia (Fig. 3.11). However, the later events in the adult leg

patterning, namely the elaboration of the patterning in the tarsal regions, were incomplete. Bab was expressed as a single band in the tarsus while the *dac* expression domain did not extend into the tarsus (Fig. 3.11 top) and did not overlap with that of Bab (Fig. 3.11 top). During pupal leg development these larval patterns matured into the conserved adult patterns (Fig. 3.11 bottom). The Bab expression domain resolved into four stripes of strong expression (corresponding to the putative tarsal joints) flanked by areas of low expression (Fig. 3.11 bottom). *dac* was now expressed in the proximal tarsus and overlapped with the proximal-most Bab expression domain (Fig. 3.11 bottom). At this time, Bab expression retracted from the proximal part of Ta1 and a new domain of expression appeared in the trochanter.

In *Drosophila*, the transition of Bab and Dac expression patterns observed during *Manduca* metamorphosis occurs during the course of leg imaginal disc patterning during the mid to late last larval instar (Fig. 3.2A; Chu et al. 2002). In the cricket, *Acheta domestica*, a similar change in Bab expression was also observed during embryonic development (Fig. 3.2B; Erezyilmaz et al. 2004).

Based on these data, I suggest that the single-segmented tarsus in *Manduca* larva is the result of truncating the conserved general patterning system of the adult leg. In most holometabolous insects, the larval tarsi are single-segmented as seen in *Manduca* and are subdivided into multiple segments in adults. This strongly suggests that this morphology evolved in the common ancestor of the Holometabola by prematurely arresting the adult leg patterning system in the hemimetabolous ancestor.

Changes in the regulation of the PD genes during metamorphosis

Based on the morphological observations and the labeling experiments, we know that the primordia in each larval leg segment contribute to more distal segments in the adult leg, suggesting that the cells in the primordia change their segmental identities during metamorphosis (Fig. 3.1; Tanaka and Truman 2005; Kim 1959; Miles and Booker 1993). Consistent with these data, during the last larval instar, these cells also

changed their PD gene expression according to their future segmental positions (Fig. 3.8,9). Hence, *Dll* appeared *de novo* in the primordium in the larval femur, which gives rise to the adult tibia. In insect legs, *Dll* is usually expressed in the tibia, but not in the femur (Fig. 3.2). Similarly, Bab was expressed *de novo* in the primordium in the larval tibia, which gives rise to the adult tarsus (Fig. 3.9). Bab expression is unique to the tarsal segments. This latter primordium also turned off *dac* in the distal side, which apparently gives rise to the distal part of the adult tarsus. Generally, *dac* is not expressed in the distal tarsus in insect leg (Fig. 3.2). In contrast, the primordium in the larval trochanter did not turn off *Dll* and Exd expression during metamorphosis, which resulted in the pupal femur expressing these genes in most of the segment.

The observed modifications in the expression of the PD genes suggest changes in the regulatory interactions among these genes during metamorphosis. The clearest example is the change in the relative expression patterns of *dac* and Bab. The expression domains of these two genes during larval leg development were mutually exclusive and indicated an antagonistic, most likely indirect, interaction. In *Drosophila*, ectopic *dac* can suppress *bab* expression in the tarsus and loss of *dac* results in derepression of *bab* (Chu et al. 2002). During metamorphosis, however, the two domains overlapped in *Manduca*, suggesting a modification in the mutual regulation of the two genes. A similar domain overlap is also eventually established in the leg of *Drosophila* (Fig. 3.2A bottom).

Another prominent modulation in the PD gene expression observed was that of Bab. In the leg from early prepupae, Bab domain showed four stripes of strong expression flanked by low expression domains (Fig. 3.5C). The equivalent modulation of *bab* expression takes place in *Drosophila* leg imaginal disc and requires the presence of *dac* in the *bab* expression domain. (Chu et al. 2002). The hypomorphic mutant of *dac* lacks down-regulation of Bab and fails to produce Ta1-Ta3 and their joints (Chu et al. 2002). This mechanism may or may not be present in the developing pupal leg of *Manduca*. In *Drosophila dac* and *bab* overlap is more extensive, spanning the distal Ta1, the Ta2 and the proximal Ta3 (Kojima 2004), while in *Manduca*, the overlap between the two domains is localized in the distal Ta1 and, probably, the proximal Ta2.

Therefore, based on the extent of its expression, *dac* may not be able to down-regulate Bab expression in the distal Ta2 and Ta3 in *Manduca*.

Another gene that plays a role in the modulation of *bab* expression in *Drosophila* is *bowl* (de Celis Ibeas and Bray 2003). *bowl* is expressed in the border between the femur and tibia and Ta5 and pretarsus (de Celis Ibeas and Bray 2003). It is required non-autonomously for lowering the expression level of *bab* in the proximal and distal segments of the tarsus and up-regulating *dac* in the proximal tarsus. *bowl* might also be contributing to modulation of *bab* expression pattern in *Manduca* and its expression should be examined in the future.

Molecular mechanisms responsible for changes in more proximal segments

Although I found changes in expression patterns of the PD genes, *dac* and Bab, that may be responsible for differences between the larval and the adult tarsi, the patterning mechanism responsible for the changes in the tibia and femur is not clear. Especially in the tibia, the expression patterns of the two PD genes, *Dll* and *dac*, are identical between the two stages. Even in *Drosophila*, no additional PD patterning gene that is specifically expressed in the tibial segment has been found. Yet, the adult femur and tibia develop elaborate morphological features along the PD axis including their overall shape and structures like the tibial spurs.

There are two potential molecular mechanisms underlying the transformation of the tibia and the femur. One is the expression of a Hox gene, *Antennapedia* (*Antp*). In *Drosophila*, *Antp* is expressed in all three pairs of the leg imaginal discs. During embryogenesis, it is expressed in the entire discs to specify the leg fate, but during the larval development, the expression gradually retracts to the proximal femur (Emerald and Cohen 2004). Retraction from the tarsus is required for proper tarsal development. However, it is needed for proper development of femur and tibia until later in development. One of *Antp* functions is to prevent the coexpression of *hth* and *Dll* in the proximal femur, which would transform it to antenna. Interestingly, the proximal adult

femur of *Manduca* co-expresses *Dll* and nuclear Exd, which is localized by Hth. In the embryonic legs of *Manduca*, Antp protein is expressed in the whole leg until 50% E (Zheng and Booker 1999). Conceivably the distal border of Antp expression retracts more proximally (to tibia or femur) during metamorphosis to provide an additional positional information for the proximal leg segments.

Morphological changes in the femur and tibia are mainly due to differential growth and do not involve a new round of segmentation, thus molecular players involved in the leg tissue growth in *Drosophila* may be another good candidate. Many molecules involved in leg growth along PD axis have been characterized in *Drosophila.* These genes are often expressed in the leg as concentric rings. One set of such genes are those involved in specifying the joints. These include *Notch* and genes encoding the ligands, *Serrate* and *delta*, and the modifier of Notch activity *fringe* (de Celis 1998; Rauskolb and Irvine 1999). Mutant phenotypes of these genes in the leg include fusion of segments and reduced size. *Sp1* in *Drosophila* and its homolog *Sp8* in *Tribolium* are also expressed in segmentally reiterated patterns and are involved in growth of the leg (Estella et al. 2003; Beermann et al. 2004). Lack of their function in both *Drosophila* and *Tribolium* results in adult leg segments that are fused and reduced in size.

Another group of molecules important in controlling growth as well as patterning is the secreted signaling factors Dpp, Wg and Hedgehog (Day and Lawrence 2000). As these proteins are morphogens, the shape of their concentration gradient across the tissue can influence the pattern of growth. It is possible that during the pupal leg development, expression patterns of these factors are modified to control morphogenetic growth. Alternatively, the initial changes in the size and shape of the developing pupal leg could change the distribution of these morphogens, which would then modify further growth of different regions within the leg.

Relationship between the larval and adult leg segments

My previous analysis showed that the cells in the adult primordia shift their identities to those of more distal segments in the adult leg. Thus, except for the coxa and the trochanter, the corresponding segments between the larval and adult legs are not made up of the same epidermal cells and their descendants (Fig. 3.1D; Tanaka and Truman 2005). In contrast, in terms of the PD gene expression patterns that characterize them, the corresponding segments appeared to share more in common. Both the adult and the larval tibia and, probably, trochanters were defined by exactly the same combination of PD genes (Fig. 3.11). In case of the adult femur and tarsus, they were also defined by the same PD genes as in the larva, but had an additional PD gene expressed in the proximal regions (Fig. 3.11). Thus, it appears that while the corresponding segments in the two legs do not have to derive from the same cells, their molecular identities defined by the PD genes, have to be similar across the two stages.

One possible reason for the requirement of the larval and adult segments to retain similar molecular identities is for the development of the muscles and the motor neurons in the adult leg. During metamorphosis the larval muscles in the leg degenerate and most of the adult muscles are derived from the imaginal myoblasts located near the adult primordia (Consoulas et al. 1997). In contrast, many of the larval motor neurons in the leg persist through metamorphosis to innervate the adult muscles (Kent and Levine 1988). Based on the finding that the femoral flexor muscle in the coxa is innervated by the same motor neuron in the larval and the adult legs, it is thought that some motor neurons innervate muscles in the same segment in the adult and the larval legs (Kent and Levine 1988). As both muscles and neurons are thought to use the epidermis as an important source of positional information during their migration (Consoulas et al. 1997), it may be necessary for the larval and the adult leg segments to retain the same molecular identity.

Another possible reason for the maintenance of the similar PD gene expression pattern in each segment is the placement of the segment boundaries in the adult leg. Examination of Figure 3.1D suggests that many of the segmental boundaries in the adult

leg are established anew. In *Drosophila* leg imaginal disc, the segment boundaries (or joints) are positioned by activating the joint patterning genes such as *Notch*, *delta* and *Serrate* in each segment just proximal to the future joints (Rauskolb 2001). It is thought that these genes possess distinct enhancers for their expression in each segment, whose activity is regulated by different combinations of the PD genes (Rauskolb 2001). If the same set of enhancers is used for positioning the joints in both the larval and the adult leg, then having the similar PD expression in each of the larval and the adult segments would be essential for proper joint development.

Decoupling of cellular and molecular identities of leg segments in *Manduca* has probably evolved, because of the complex mode of leg development involving the adult primordia. In a member of more basal group such as the beetle *Tenebrio molitor,* the larval leg does not appear to possess a discrete set of cells similar to the adult primordia (Huet and Lenoir-Rousseaux 1976). Rather, all the epidermal cells of the larval leg appear to contribute to the adult leg and the segmental boundaries appear to be maintained through metamorphosis. In this case, one may observe both the cellular and molecular continuities between the larval and the adult leg segments.

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I thank Dr. Richard Vogt for supplying *Manduca Dll* clone, Dr. Robert White for Exd antibody, Dr. Frank Laski for Bab antibody, Dr. Gerard Campbell for Al antibody and Dr. Sean Carroll for sharing the sequence for *Précis coenia dac.* Brigid O'Donnell shared the protocol for the section *in situ* hybridization. I also would like to thank Dr. Horacio de la Iglesia for use of his cryostat. This study was supported by funding from NSF to JWT.

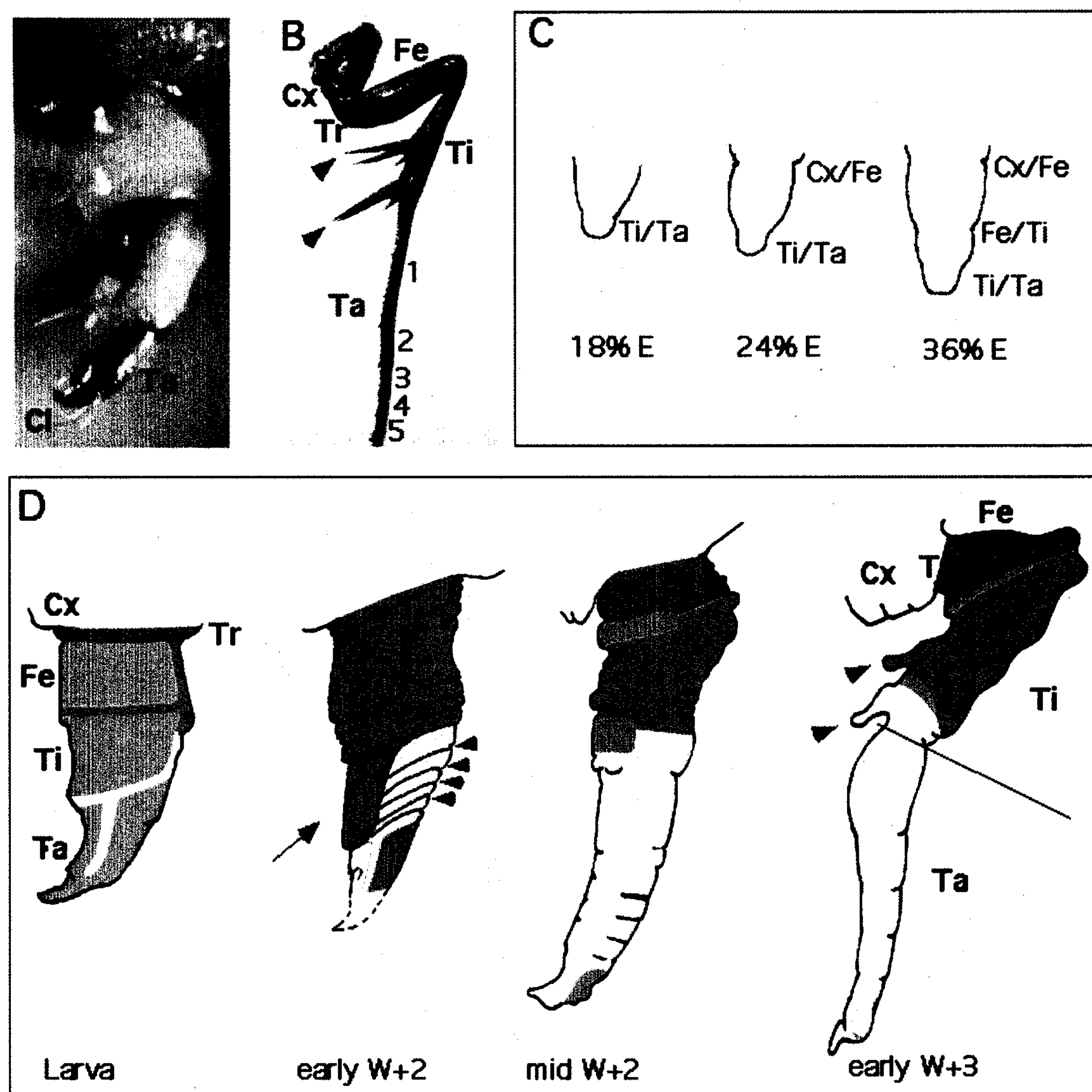
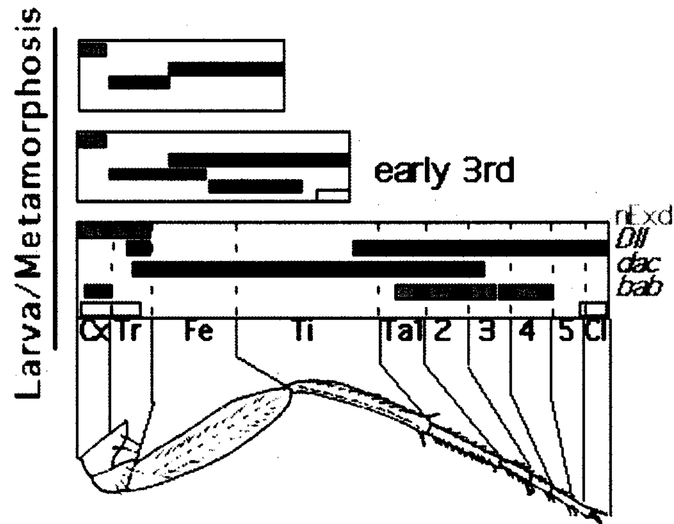


Figure 3.1. Leg development in *Manduca.* (A) Leg of a 5th instar larva. (B) The metathoracic (T3) leg of the adult. Two pairs of spurs are present on the tibia (arrowheads) and the tarsus (Ta) is divided into 5 subsegments (Ta1-5). The leg is approximately seven times longer than the 5th instar leg. (C) Diagram of embryonic leg development showing the appearance of constrictions that mark the boundaries of the leg segments. % E, the percent of embryonic development. (D) Schematic summary of the morphogenesis of the T3 adult leg during the prepupal stage. The adult primordia are shown in colors and the larval regions are in grey. See text for details. In the early W+2 leg, the distal region drawn with dashed line is an estimate as this part is covered by the larval cuticle at this stage. The larval region in the tibia (arrow) hangs over the ventral adult primordium in the tibia. The latter shows a sign of segmentations, which will give rise to adult tarsal segments (four arrowheads). In early W+3, the line indicates presumptive Ti/Ta border. Two arrowheads point to the tibial spurs. W, wandering; Cx, coxa; Tr, trochanter; Fe, femur; Ti, tibia; Ta, tarsus; Cl, ptretarsal claw.

A. Fly



B. Crickets

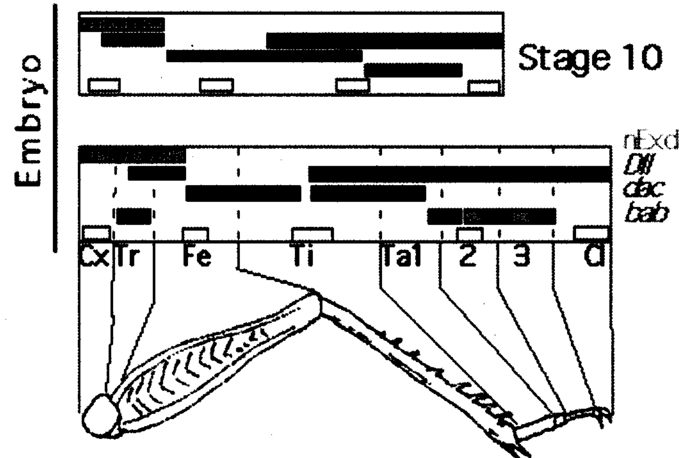


Figure 3.2. Molecular patterning of the PD axis in *Drosophila* and crickets. In *Drosophila* (A), the earliest patterning events, namely, the activation of *Dll* and nuclear Extradenticle occurs during embryogenesis. The patterning resumes during the larval instars. In the early 3rd instar, the leg disc has yet to achieve the final expression pattern of the PD genes. *bab* is still expressed as a single band and its expression domain does not overlap with that of *dac*. In the late 3rd instar the mature expression pattern is attained. In crickets (B) (*Gryllus bimaculatus* and *Acheta domesticus*), the entire patterning process takes place during the embryogenesis. *bab* expression pattern goes through a similar transition as the one observed in the fly (Erezyilmaz et al. 2004). Based on the available data from the literature, an overlap between *bab* and *dac* domain do not appear to be present, but it has not been determined (Erezyilmaz et al. 2004; Inoyue et al. 2002). *bab* expression pattern is estimated from the expression pattern of *bab* protein product. Cx, coxa; Tr, trochanter; Fe, femur; Ti, tibia; Ta, tarsus; Cl, ptretarsal claw.

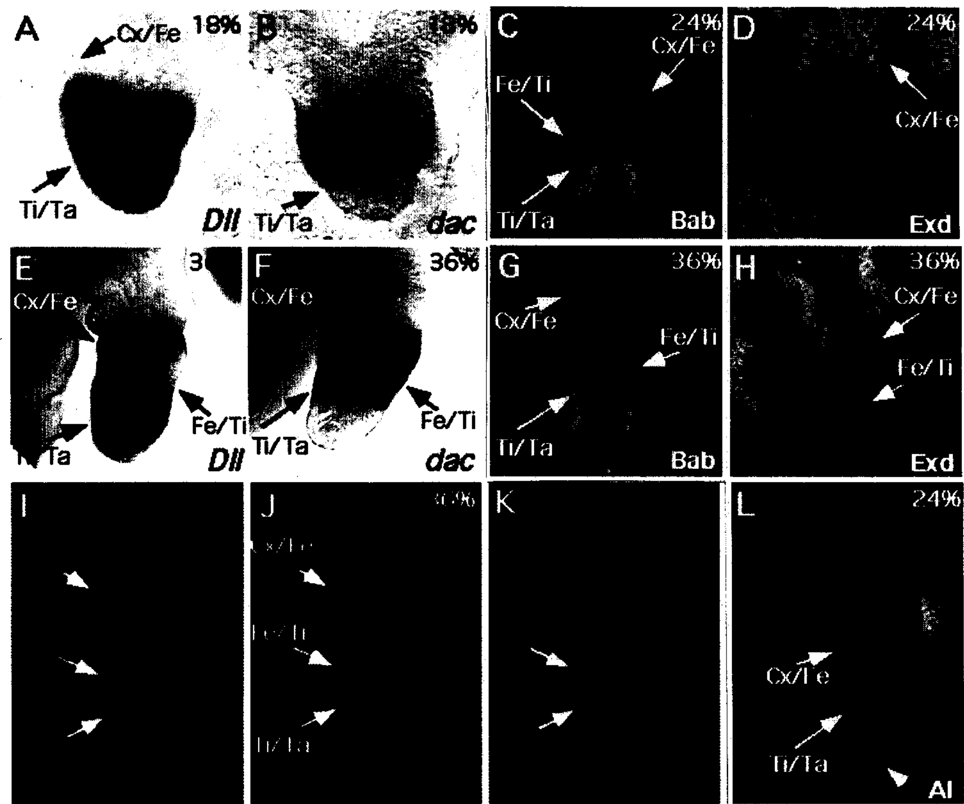


Figure 3.3. Expression patterns of the PD genes in the larval leg during early embryonic development. (A, E) *Dll in situ* hybridization showing expression at (A) 18% E. (E) 36% E. (B, F) *dac in situ* hybridization showing expression at (B)18% E. (F) 36% E. (C, G) Bab immunoreactivity in confocal section at (C) 24% E. (G) 36%. (D, H) Exd immunoreactivity in confocal section at (D) 24% E. (H) 36% E. (I-K) Confocal section through a 36% E leg showing (I) *dac in situ* hybridization and (K) Bab immunoreactivity. (J) Merge of the two channels showing the sharp boundary between *dac* and bab expression domains. The weak staining in the Fe/Ti area of (K) is an artifact from the combined procedure (compare with G). (L) Al expression in 24% E leg. Arrowhead points to a strong expression in the body wall. Cx, coxa; Fe, femur; Ti, tibia; Ta, tarsus.

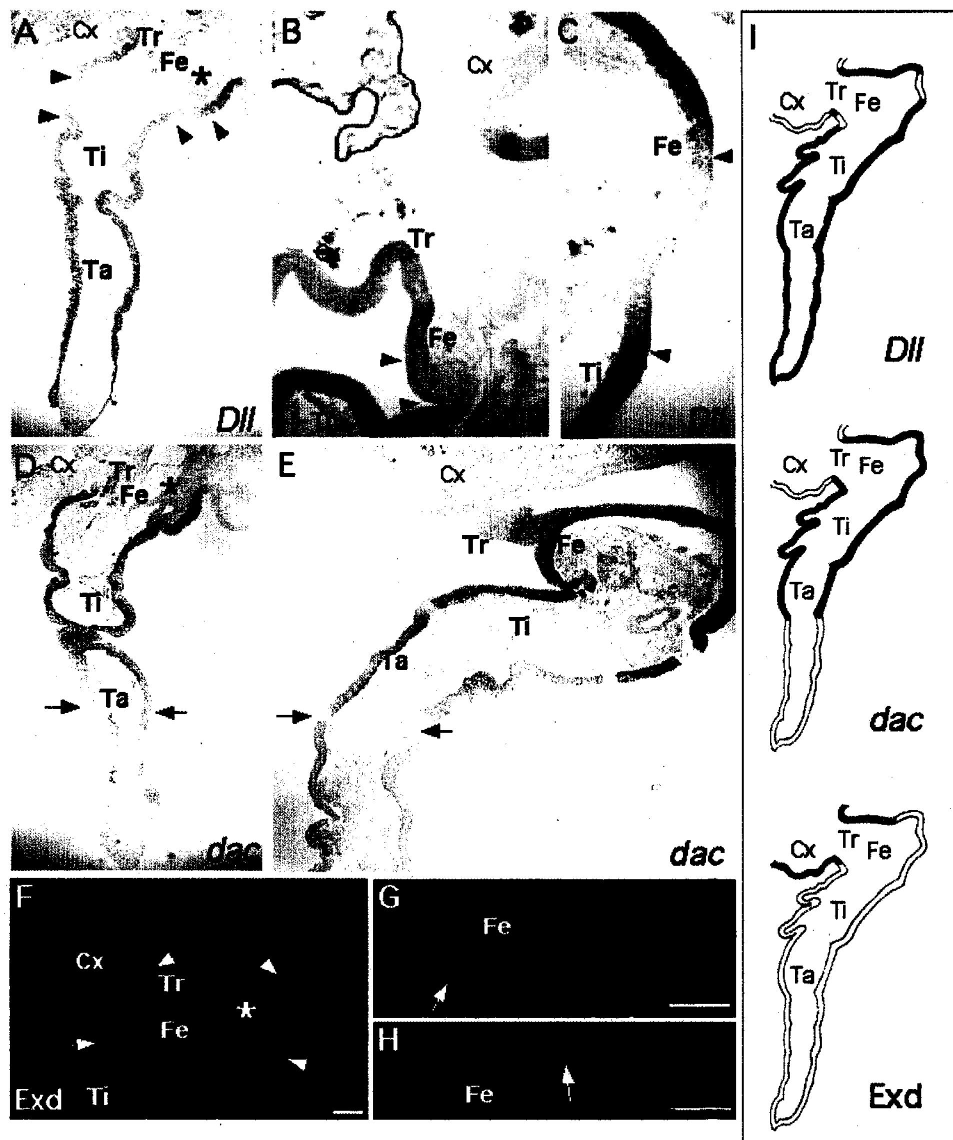


Figure 3.4. Expression patterns of *Dll* and *dac* mRNA and nuclear Exd during adult leg development. (A-C) *Dll* expression. (A) In the mid W+2 leg, *Dll* is expressed in the whole leg except in the small area derived from the larval femur (between the arrowheads) and in the presumptive coxa (area proximal to the trochanter). (B, C) Proximal region of the early W+3 leg to show the persistence of the *Dll* mRNA free areas on the ventral (B) and dorsal (C) side respectively. (D, E) *dac* expression. (D) In the mid W+2 leg, *dac* is expressed from the trochanter/femur border to the proximal tarsus. (E) This pattern is maintained in the early W+3 leg. (F) Nuclear Exd signals in the mid W+2. Strong signal is observed proximal to the presumptive coxa/trochanter border (arrowheads). Weak signal is present in the femur (proximal to the arrows). (G, H) Distal limits of nuclear Exd in the ventral (G) and dorsal (H) femur in the early W3 (arrows). (I) Schematic diagrams showing the expression patterns in the early W+3 leg. Asterisks in (A) and (D), invaginating apodeme. *Dll* and *dac* mRNA expression is missing from this structure. Ventral is to the left. Cx, coxa; Tr, trochanter; Fe, femur; Ti, tibia; Ta, tarsus.

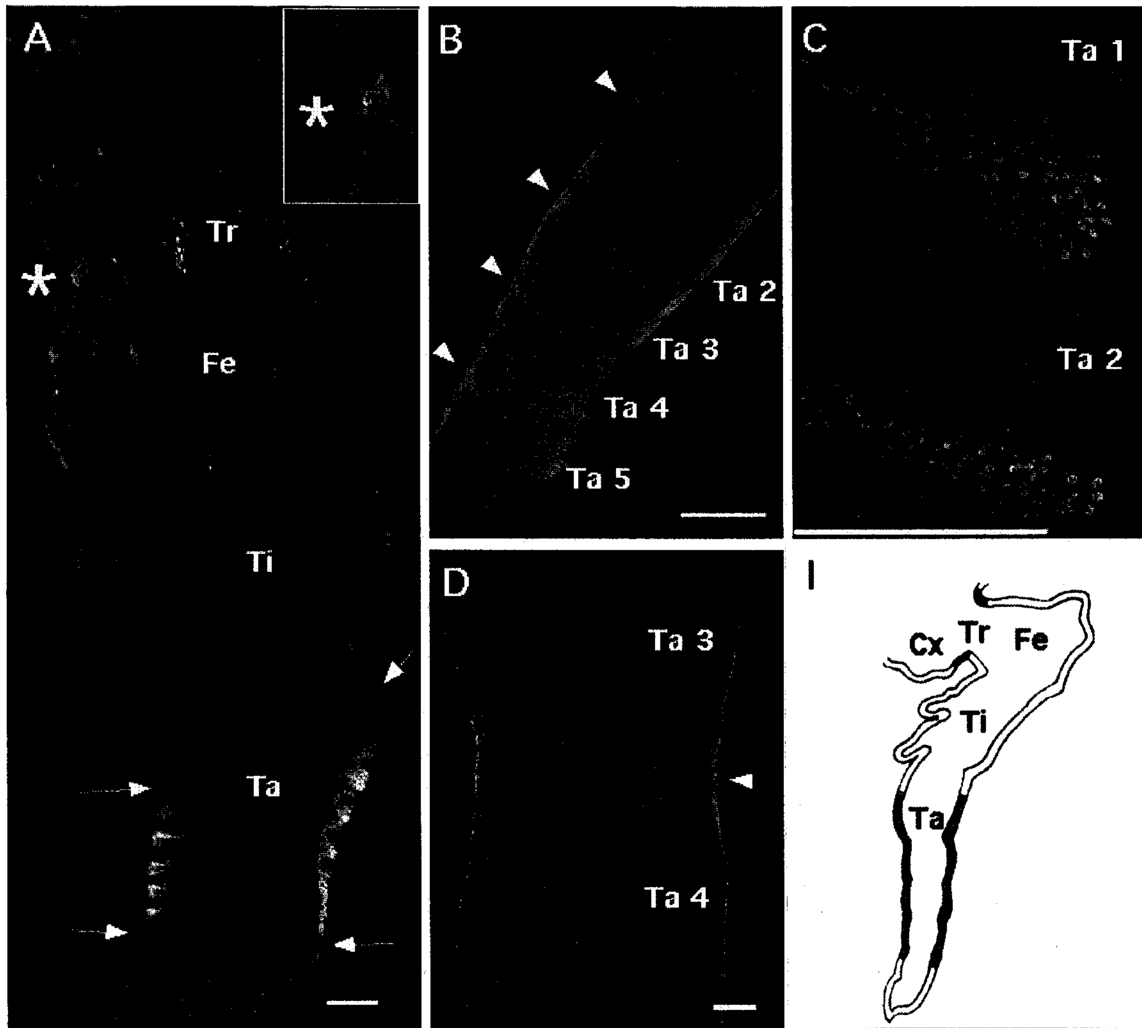


Figure 3.5. Bab expression during the pupal and adult leg development. (A) Overall view of a confocal optical section through a W+2 leg. A new expression domain appears in the ventral putative trochanter (asterisk). Arrows point to the borders of Bab expression domain in the tarsus. (B) Tarsal segments in the late W+2 leg. The segment is more extended as compared to (A), and Bab shows rings of strong expression (arrowheads). (C) Magnified view of early W +2 leg. Difference in the expression levels in and between the rings are already visible at this stage. (D) Ta3 and Ta4 in the P+4 leg. The ring is now localized to the presumptive joint of the adult leg. Vertical rows of strongly labeled nuclei belong to the scale precursor cells. (I) Schematic representation of Bab expression in the early W+3 leg. Cx, coxa; Tr, trochanter; Fe, femur; Ti, tibia; Ta, tarsus. Scale bar = 100 um.

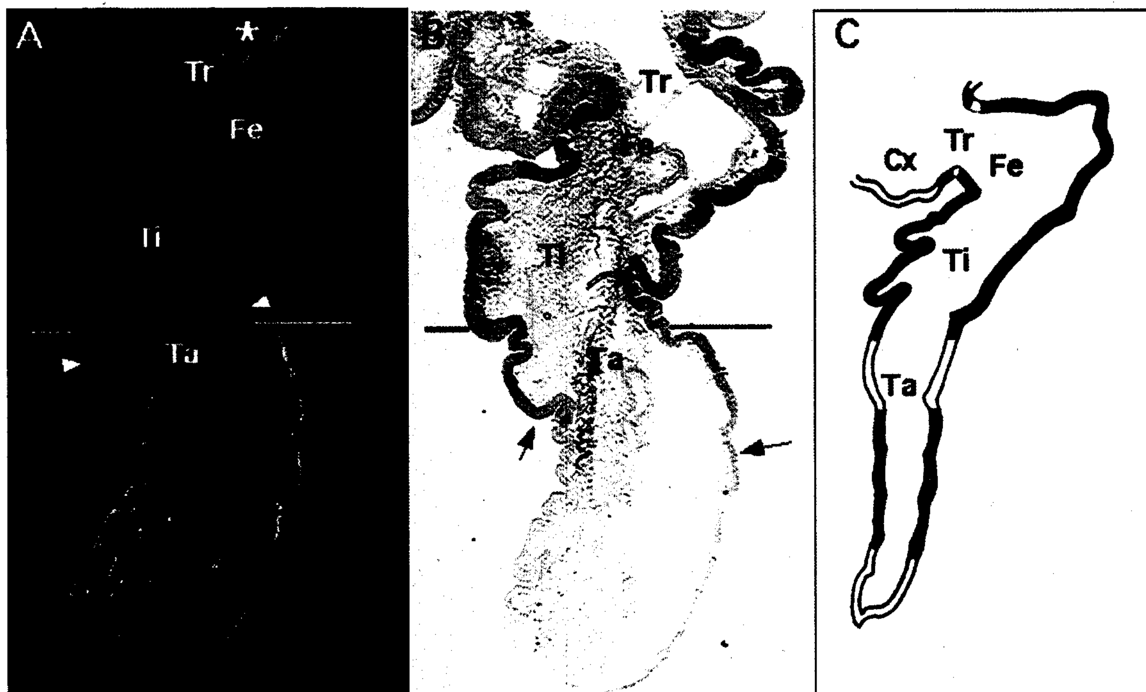


Figure 3.6. *dac* and Bab domains overlap during the pupal leg development. (A) Fluorescent immunocytochemistry with Bab antibody on mid W+2 leg frozen section. Arrows show proximal border of Bab expression domain in the proximal tarsus. Asterisk, Bab expression domain in the trochanter. (B) *dac in situ* hybridization on an alternate section. Arrows, distal borer of *dac* expression domain. Lines show the same level along the PD axis. (C) Schematic representation of *dac* and Bab expression patterns in the early W+3 leg. Red, *dac*; Green, Bab. Yellow, areas of co-expression. Cx, coxa; Tr, trochanter; Fe, femur; Ti, tibia; Ta, tarsus.

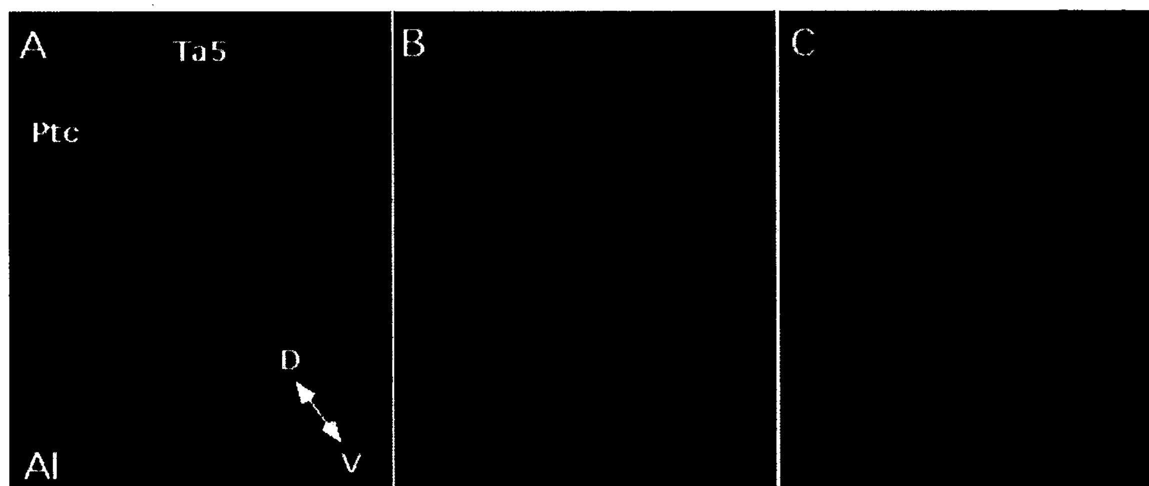


Figure 3.7. Aristaless is expressed strongly in the developing adult claw. (A) Al expression in the pretarsal claw of a P+6 leg. Strong signal is detected dorsally. Projection of a confocal Z stack shown. Ptc (pretarsal claw). (B) A single optical section of Al immunoreactivity from the same leg to show that the signal is coming from the epidermis. (C) Expression in (B) merged with the propidium iodide channel (red color).

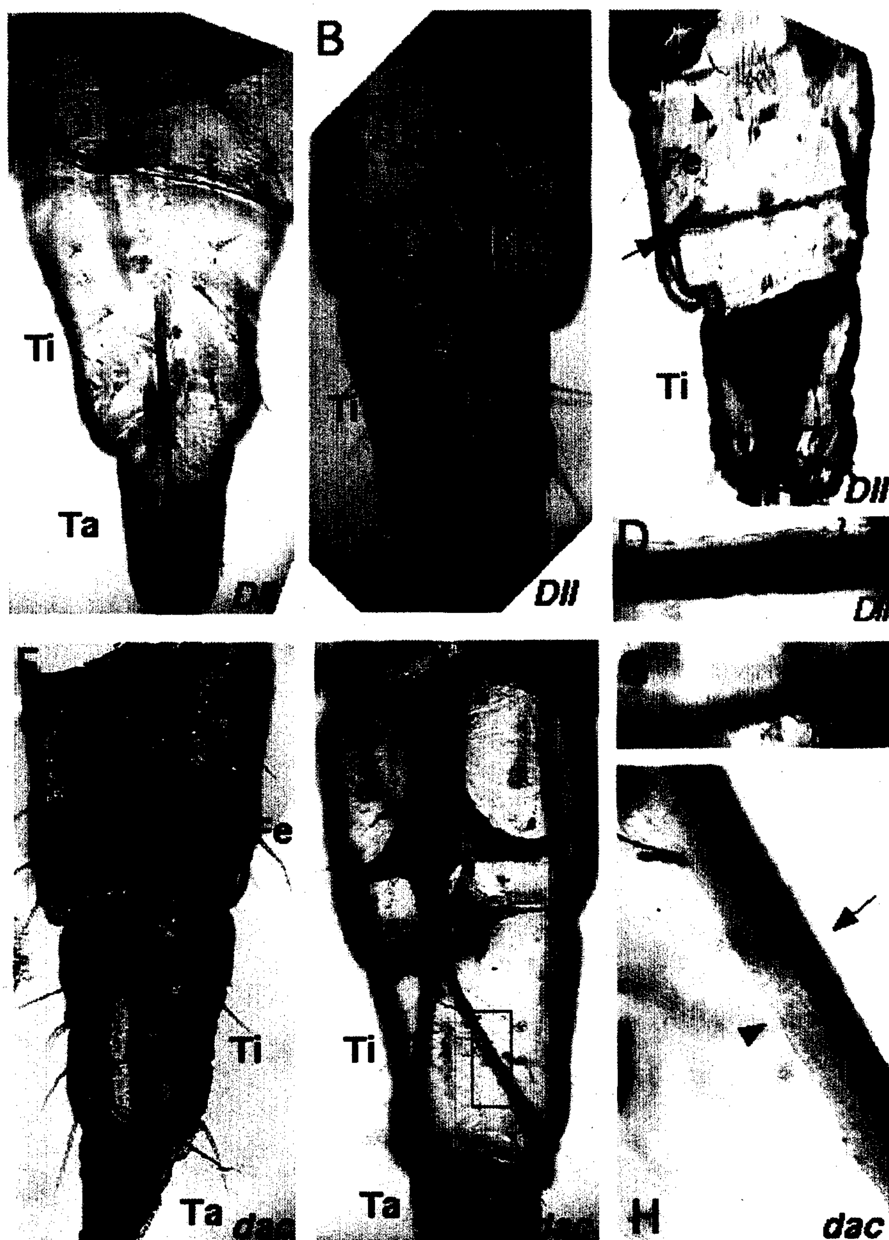


Figure 3.8. *in situ* hybridization showing *Dll* and *dac* expression in the developing adult primordia. (A-D) *Dll* expression. (A) *Dll* expression is not detected in V+2 hr leg. Dorsal side is shown. (B) In the V+3 leg, *Dll* is detected in all the primordia, but the expression is missing from the proximal half of the femoral primordium on the dorsal side (arrow). The primordium in the trochanter is not visible in this image. (C) Ventral V+3 leg showing expression in the primordia in the trochanter (arrowhead), the femur (arrow) and the tibia (asterisk). (D) Magnified view of the ventral femoral primordium. *Dll* is not expressed in the proximal side (bracket). (E-H) *dac* expression. (E) V+ 2 hrs dorsal side. There is a strong ubiquitous staining. (F) V+3 leg dorsal side. Expression is detected in all the primordia including the one in the trochanter (arrowhead). (G) Magnified view of the primordium in the trochanter to show *dac* expression. (H) Magnified view of the tibial primordium (marked area in (F)). *Dac* expression is strong on the proximal side (arrow), but lacking from the distal side (arrowhead). Fe, femur; Ti, tibia; Ta, tarsus.

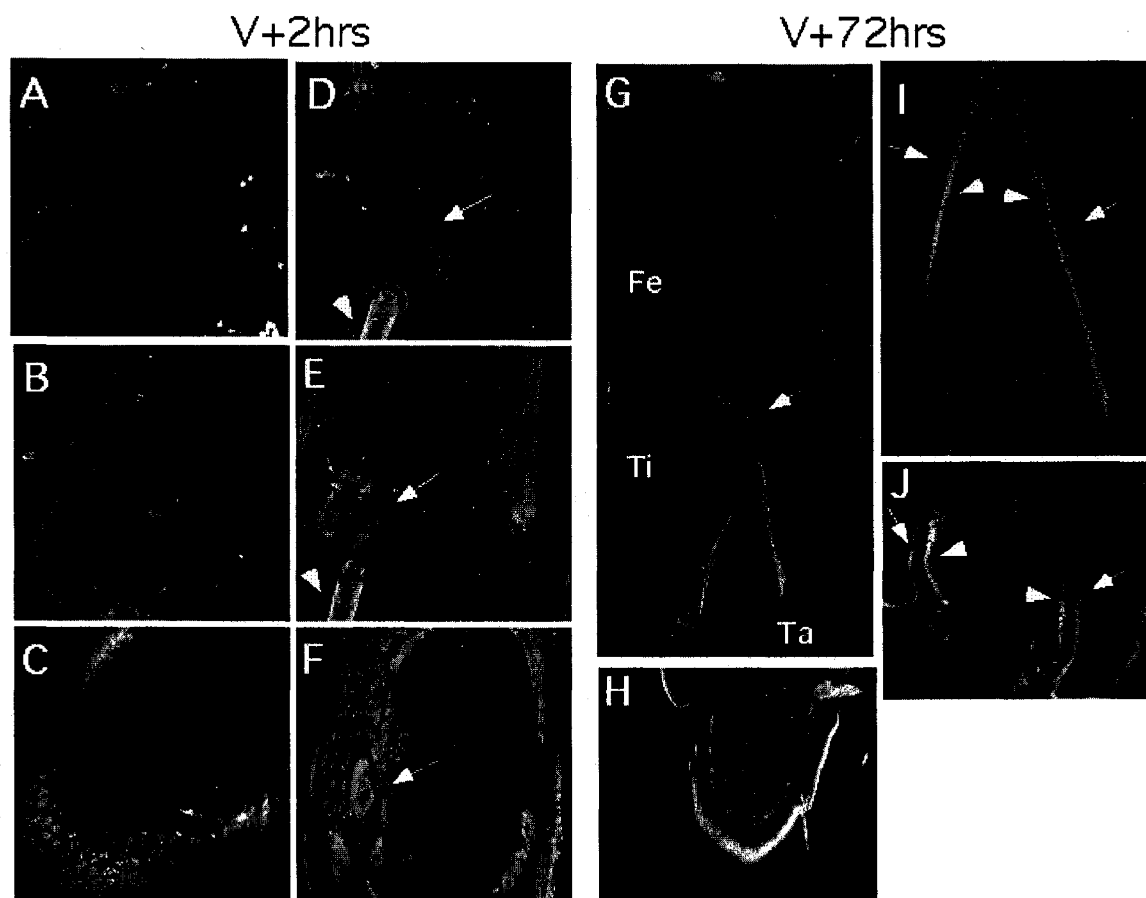


Figure 3.9. Confocal sections showing Bab expression in the developing adult primordium. (A-F) V+2 hrs leg. (A-C) dorsal side. (D-F) ventral side. There is a strong expression in the tarsus (C, F), weak, but clear expression in the tibia (B, E), and very weak or no expression in the femur (A, E). Giant bristle nuclei showed Bab signal (D, E, F; arrows). Arrowheads, autofluorescent bristles. (G-J) V+3 leg. (G) Bab is strongly expressed in the primordium in the tibia (G) and the larval regions in the tarsus (H). Arrow, proximal limit of Bab expression in the primordium. (I, J) Magnified view of the tibial primordium. Within the primordium, Bab expression is confined to the distal region (arrowheads) and absent from the proximal part (outer folds; arrows). Dorsal (I) and ventral (J) aspects of the primordium in the tibia are shown. Fe, femur; Ti, tibia; Ta, tarsus.

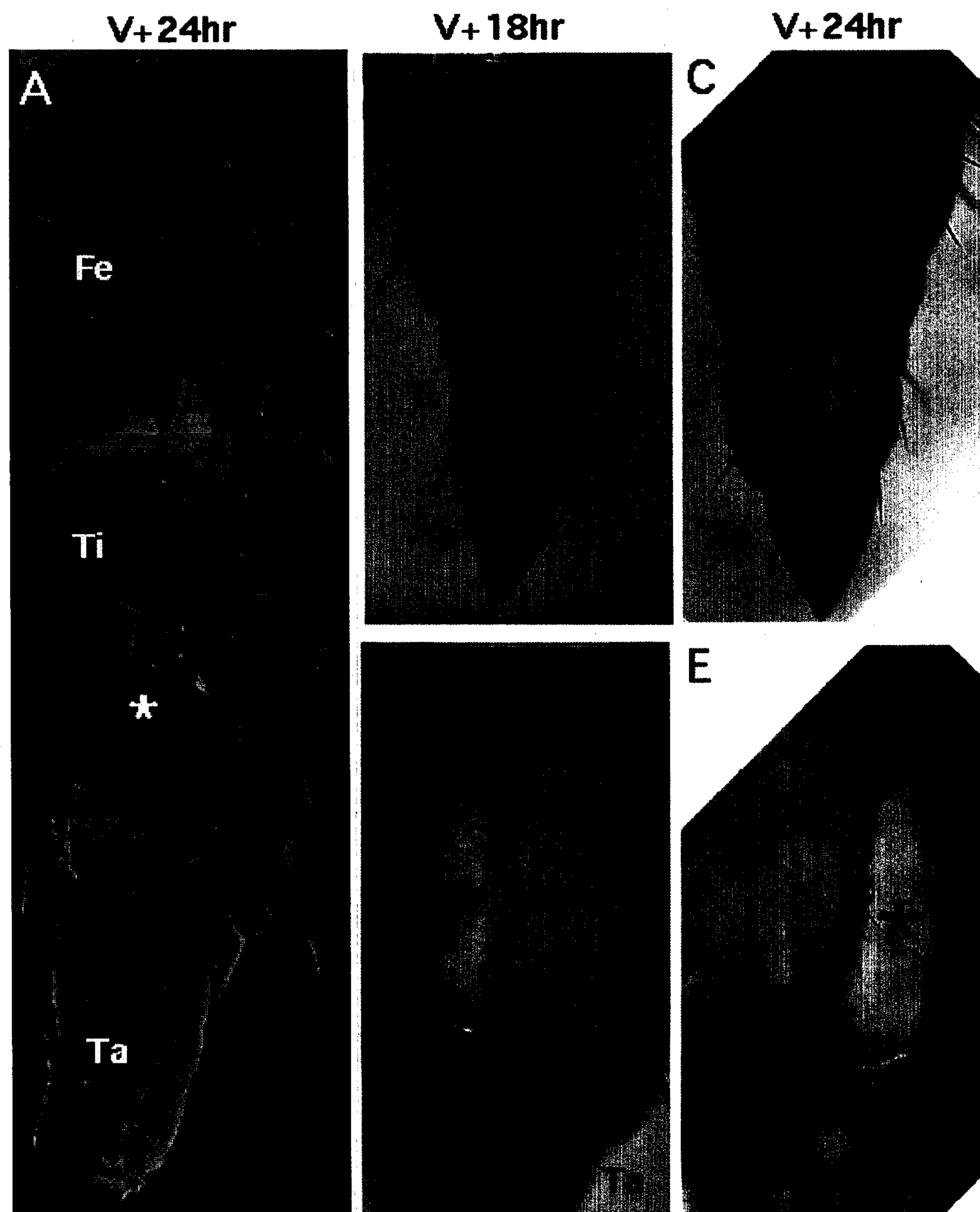


Figure 3.10 *Dll* expression in the primordia precedes cell proliferation. (A) Confocal image of V+ 24hrs leg immunostained for PH3 (dorsal view). No PH3 signal is detected at this stage. Only the autofluoresence from the cuticle is visible. (B, D) V+18 hr leg (dorsal view), *Dll* is already expressed in the tibial primordium. The regions of the primordium along the Ti/Ta boundary (arrowhead) initiate proliferation first. Proliferation then spreads along the sides (arrow) of the muscle attachment site. (D) shows an enlarged view of the primordium. (C, E). V+ 24 hr leg (dorsal view). The area of the primordium with *Dll* expression expanded in V+24 hrs leg. In (C), the right side is hidden by the overlying cuticle. Arrow, Asterisk, muscle attachment site lacking the epidermis. Fe, femur; Ti, tibia; Ta, tarsus.

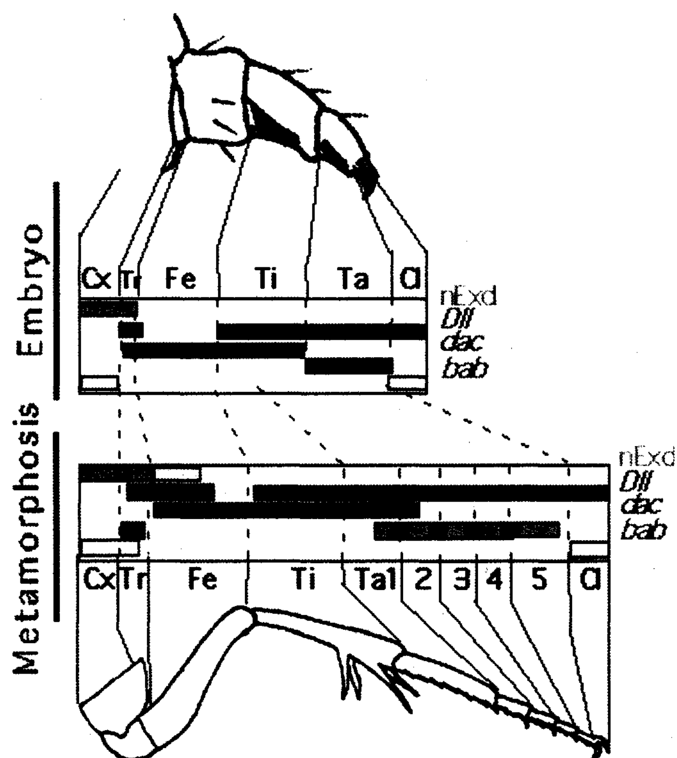


Figure 3.11. Molecular patterning of the PD axis of the larval and adult legs in *Manduca*. The PD gene expression pattern at the end of the larval leg development is reminiscent of the *Drosophila* in the early 3rd instar. I propose that this premature pattern is used to pattern the PD axial pattern of the larval leg. During metamorphosis the patterning process is resumed to produce the mature pattern similar to those in the adult legs of the fly and the crickets. *bab* and *al* gene expression patterns are based on the expression patterns of their protein products. For detail, see text. Cx, coxa; Tr, trochanter; Fe, femur; Ti, tibia; Ta, tarsus; Cl, ptretarsal claw.

Chapter 4

Role of the Hox protein Sex combs reduced in segment-specific differentiation of the thoracic legs

Introduction

Appendages on body segments of insects have specialized morphologies adapted for specific functions and allow them to manipulate different environmental factors. Each pair of ventral appendages on the three gnathal segments has a unique morphology that contribute to forming the feeding mouthparts, whereas the ones on the three thoracic segments are adapted for locomotory functions and, in some cases, for predation. Insects with holometabolous life cycle switch their ecological niches at metamorphosis. Therefore, the functional requirements on segmental specializations are also expected to change, depending on particular life stages. All three thoracic segments in the larvae usually have pairs of legs that are essentially identical. In the adults, however, each pair may develop a segment-specific morphology needed for its adult function. This divergence of leg function at metamorphosis is also evident in the tobacco hornworm *Manduca sexta.* The three pairs of the larval thoracic legs show no differences in size, shape and positions of major bristles. In contrast, the adult legs display several segment-specific characteristics including size and accessory structures.

What is the developmental mechanism that allows the animal to produce morphologically uniform larval legs, but then differentiate them in a segment-specific manner at metamorphosis? Obvious molecular candidates are the homeodomain transcription factor Hox genes, which are expressed in specific sets of segments and direct developmental programs unique to those segments (Hughes and Kaufman 2002). In *Drosophila*, Hox gene mutations that cause abnormal expression result in transformation of one segment into another segment (Struhl 1982). Hox gene mutants in the lepidopterans *Manduca sexta, Bombyx mori* and the beetle *Tribolium castaneum* and RNAi knockdown experiments in the bug *Oncopeltus fasciatus* demonstrated that Hox genes play similar developmental roles across insect orders (Miles and Booker

1993; Nagata et al. 1996; Curtis et al. 2001; Hughes and Kaufman 2000). This dramatic effect of Hox gene misexpression initially led researchers to think that Hox genes sit on top of genetic hierarchy and regulate a few genes that, in turn, control a large number of downstream genes (Mann and Morata 2000). However, further studies have discovered that Hox genes also act as micromanagers of development in insects (Roch and Akam 2000; Rozowski and Akam 2006). In this role, Hox genes would be turned on at precise time points, at appropriate levels, and in specific locations within segments to regulate fine-scale developmental events such as appendage growth and bristle formation. (Stern 1998, 2003; Mahooz et al. 2004; Rozowski and Akam 2006). Thus, Hox genes can regulate genes at multiple levels of developmental cascade through insect ontogeny.

Control of segmental identities in the thoracic legs has been studied extensively in *Drosophila*. Three Hox genes, *Sex combs reduced* (*Scr*), *Antennapedia* (*Antp*) and *Ultrabithorax* (*Ubx*) specify the thoracic segments (Struhl 1982; Cohen 1993). *Antp* is expressed in all pairs of thoracic appendages (T1-T3) and specifies the "leg" fate (Struhl 1982; Emerald and Cohen 1993). *Scr* is expressed in the T1 segment to suppress wing development and control the T1 leg development, while *Ubx* is expressed in the T3 pair of legs to produce T3-specific features (Cohen 1993; Glicksman and Brower 1988). The default T2 leg fate results from the absence of inputs from *Scr* and *Ubx* (Cesares and Mann 2001).

Hox gene expression during the larval leg development in the Holometabola has been analyzed in only a few species. Antp is expressed in the embryonic legs of the three lepidopteran species that have been examined (*Manduca,* Zheng and Booker 1999; *Bombyx*, Nagata et al. 1996; *Precis coenia,* Warren et al. 1994). Interestingly, however, Ubx protein in *Manduca, Tribolium,* the lacewing *Chrysoperia carnea* embryos is expressed in the body wall, but not in the legs of the T3 segment (Zheng et al. 1999; Mahooz et al. 2004). This observation led Mahooz et al. (2004) to propose that the homonymous larval legs of Holometabola result from the lack of *Ubx* expression in the leg. Similarly, Scr protein expression in *Bombyx* and *Tribolium* embryos (Kokubo et al. 1997; Curtis et al. 2001) is limited to the T1 body wall and excluded from the legs.

These findings lead me to hypothesize that the Hox genes *Scr* and *Ubx* are not activated in the T1 and T3 legs of *Manduca* until metamorphosis when they divert the adult development of these legs from the default T2 pathway.

In order to test this hypothesis, I decided to study the expression patterns of Scr protein during both larval and adult leg development using the cross-reactive antibody made against *Drosophila* Scr. In *Drosophila* leg imaginal discs, Scr is expressed weakly throughout the leg and strongly in the future anterior tarsus, tibia and peripheral regions during the larval stage (Glicksman and Brower 1988). In the pupal leg, the strong expression becomes restricted to the future sex comb location, where it is necessary for the development of this T1-specific feature (Barmina et al. 2005). In the bug *Oncopeltus fasciatus*, Scr is expressed in a patch at the tibial/tarsal border where a comb-like structure forms (Rogers et al. 1997). RNAi knockdown of *Scr* obliterates this structure (Hughes and Kaufman 2000). Scr protein and *Scr* mRNA expression has been previously examined during the embryogenesis of *Bombyx mori* (Kokubo et al. 1997). Although Scr appears to be missing from the T1 legs of the mid-stage embryo in their figure, this was not discussed by the authors.

Materials and Methods

Experimental animals

Larvae of *Manduca sexta* (L) were raised in individual containers on an artificial diet (Bell and Joachim, 1976) at 26°C under a long-day photoperiod (17L :7D). After they initiated wandering, larvae were placed in holes bored in wooden blocks for pupation. The animals were chilled on ice before dissection.

The developmental stages of animals were determined with reference to developmental transitions. The day of ecdysis to the last larval instar was designated V+0. A day after V+3, larvae began to wander (W+0). The wandering stage lasted

approximately 4 days after which the animals pupate (P+0). More precisely timed stages are indicated with hours after ecdysis (e.g. V+72 hrs or V< 2 hrs).

The embryos were allowed to develop at 26°C and staged according to Broadie et al. (1991). Ages were given as a percentage of embryonic development (%E).

Immunocytochemistry

The eggs were placed in bleach to remove the chorion, then dissected in *Manduca* saline (Riddiford et al., 1979). The embryos were fixed in 3.7% formaldehyde in phosphate-buffered saline, pH 7.2 (PBS: 130mM NaCl, 7mM Na_2HPO_4, 3mM NaH_2HPO_4) for 30 min followed by rinses in PBS with 1% Triton-X 100 (PBS-TX; Sigma, St Louis, MO). For the larvae, the legs were dissected as previously described (Tanaka and Truman 2005).

The tissues were blocked in 5% donkey or goat serum in PBS-TX for 30 min, followed by a rinse in PBS-TX. Anti-Scr was used at 1:500 in 1% PBS-Tx (Mahaffey and Kaufman 1987; Gorman and Kaufman 1995). Secondary antibodies were used at concentration of 1:1000 in 1% PBS-TX. Finally, the tissues were dehydrated, cleared in xylene, and mounted in DPX (Fluka, Buchs, Switzerland).

Image collection and analysis

All the fluorescent images were collected using confocal microscopy and processed with the NIH Image program (http://rsb.info.nih.gov/nih-image) and Adobe Photoshop (Adobe Systems Inc., San Jose, CA). To make Z-series projection of the postembryonic legs, non-epidermal areas were removed from each confocal section and then projected.

Results

Anatomy of the thoracic legs in the larva and the adult

In *Manduca sexta* the thoracic legs of the larva show no observable difference along the three segments (Fig. 4.1A). They are indistinguishable in size and shape, and the major bristles are located at the same positions on all the legs (Fig. 4.1B). The positions of smaller bristles are not stereotypical and are as variable within the leg pairs as between them.. In contrast, the adult legs display several distinct segment-specific features. The legs on the first thoracic segment (T1) have an antenna-cleaning organ called the epiphysis originating from the ventral base of the tibia (Fig. 4.1C, D). The T2 legs have a pair of spurs at the distal end of the tibia, while the T3 legs have two pairs of spurs on the tibia (Fig. 4.1C). The tibia and the tarsus on the T1 legs are significantly smaller than those in the other two pairs, while the T3 femur is smaller compared to those of T1 and T2. The difference in the size of the tarsi between the T1 and T2 segments is mostly attributable to differences in the size of the first and second tarsal subsegments (Fig. 4.1E). The other three tarsal subsegments are the same length (Fig. 4.1E).

Scr expression in the larval leg during embryogenesis

Scr protein was already detectable in the embryo at 14% E (Fig. 4.2A), when the limb buds had just become visible. Weak immunoreactivity was present throughout the embryo, while stronger Scr expression was visible in the labial segment including the nascent labial appendages. At 18% E, strong Scr expression was confined to the labial segments and the anterior T1 segment, but not in the leg (data not shown). The weak ubiquitous expression was no longer detected. At 36% E, Scr expression pattern in the body wall had expanded more posteriorly in T1 (Fig. 4.2B-F). At this stage, morphological segmentation of the thoracic legs could be recognized, which enabled us

to see the extent of Scr expression in the T1 segment. In the T1 leg, the epidermal expression of Scr was only in the anterior coxa (Fig. 4.2E), and clearly absent from the epidermis of more distal leg segments (Fig. 4.2E). There was no expression of Scr in the epidermis of T2 and T3 segments including legs. In all three pairs of leg, however, Scr was expressed in the mesodermal derivatives (Fig. 4.2E; not shown). At 48% E, the segmental differentiation of the appendages has progressed further and the embryonic cuticle was shed. The labial appendages were now fused and projected anteriorly (Fig. 4.3A, B). Scr expression in the T1 leg showed two changes. First, expression in the coxa expanded into the posterior part (Fig. 4.3C, D). Secondly, Scr was now expressed in the epidermis of T1 femur and tibia, but at a level lower than that of the body wall and the coxa (Fig 4.3C, D). This expression pattern persisted at least to 68% E when the cuticular structures such as bristles were differentiated (not shown). In summary, Scr expression was missing from the T1 leg for most of the larval leg morphogenesis but appeared at a weak level late in the development.

Scr expression during development of the adult leg primordium

Pupal development of the thoracic legs begins during the last larval instar (V). To determine the possible role of Scr during this stage, its expression pattern was examined at the very beginning of the instar before the primordia initiate growth at V< 2 hr, and then at V+72 hr, when the primordia were well developed into discs. In the V< 2 hr larva, Scr was expressed clearly in the epidermis of the dorsal tarsus and the tibia in the T1 leg (Fig. 4.4A, B). Very weak expression was also detected in the distal femur in some specimens (Fig. 4.4C). Surprisingly, a similar expression was also observed in the T2 legs (Fig. 4.4D-F), but the level of expression in the T2 leg appeared slightly lower. In the ventral half of the T1 leg, I also found that Scr expression was stronger in the more distal segments (Fig. 4.4J-L). No difference in the expression level was observed between the anterior and the posterior regions of the T1 legs.

Scr expression was strongly expressed in the developing adult primordia in the T1 leg of the V+ 72 hr larva. The increase in the expression level was restricted to the anterior sides of the primordia in the femur and the tibia, and was observed on both the dorsal and the ventral sides (Fig. 4.5A, C, G, I, K). Only weak expression was detected in the posterior half (Fig. 4.5E, G, I, K). In the femoral primordia, the expression was stronger in the distal region (Fig. 4.5A, C; compare with D). No up-regulation of Scr was detected in the primordia in the T1 trochanter or in any primordia in the T2 legs (not shown).

Scr is expressed specifically in the leg segments with T1-specific features

To follow the expression dynamics of Scr protein further in the developing pupal leg, I examined its expression at mid W+2. The leg at this stage consists of the epidermal cells derived from the adult primordia and the regions derived from the larval regions (Fig. 4.6, dotted boxes). The former region is undergoing rapid growth, while the latter is shrinking due to cell death (Tanaka and Truman 2005). In general, Scr expression was consistent with that seen at V+72 hr. Low-level expression was observed throughout the T1 leg epidermis (Fig. 4.6A-H), while strong expression was mostly localized to the regions corresponding to the areas of the discs that showed strong expression at V+72 hr. These regions were the ventral anterior side of the presumptive pupal tarsus (Fig. 4.6G, H) and tibia (Fig. 4.6C, D). In the ventral pupal tibia, the strong expression expanded into the posterior region (Fig. 4.6C). The distal end of the presumptive pupal tibia derives from the larval regions in the larval tibia, but it now showed strong Scr expression (Fig. 4.6E, F). Strong expression was not detected in the developing pupal femur (Fig. 4.6A, B), although weak expression was seen in the shrinking larval region (Fig. 4.6A, B, dotted box). In the T2 leg, the only Scr expression detected was subepidermal (Fig. 4.6I).

Discussion

Role of Scr in the T1 leg differentiation during metamorphosis

Based on the morphological uniformity of the larval legs and the lack of Ubx expression in the legs of the 50% E embryo, we hypothesized that Scr protein would not be present in the T1 leg during the embryonic development. However, I found that Scr was expressed in the presumptive leg cells during two time periods. First, prior to 18% E, there was a weak Scr immunoreactivity throughout the embryo including the cells in the T1 segment, which eventually gave rise to the leg. Between 18% E and 36% E, Scr disappeared from the developing T1 leg bud except for the coxa. However, the T1 legs began to show weak Scr expression around mid-embryogenesis after the morphogenesis of the leg was almost complete and the first embryonic cuticle was shed. The low level expression of Scr in the T1 leg was then maintained during the larval stage (Fig. 4.4). At this stage, the T2 (Fig. 4.4) and T3 (not shown) legs also showed weak Scr immunoreactivity slightly lower than that seen in the T1 leg. It is possible that this very weak immunoreactivity observed in the T2 and T3 larval legs are from Antennapedia protein as the Scr antibody was reported to recognize Antp in *Drosophila*, but with much lower affinity than Scr (Gorman and Kaufman 1995).

During the development of the adult primordia in the *Manduca* leg, Scr was up-regulated in the specific area of the developing primordia in the T1 leg (Fig. 4.5). An increase in the expression level was seen in the anterior half of the primordia located in the larval femur and the tibia and at much higher levels on the ventral side (Fig. 4.5A, C, D). At W+2 these areas were found to correspond to parts of the leg where the morphological features unique to the T1 segment develop (Fig. 4.6). No up-regulation of Scr occurred in the primordium in the larval trochanter, which gives rise to the pupal femur. The pupal femur does not differ between the T1 and T2 segments (Fig. 4.6A, B). Based on these observations, I propose that the up-regulation of Scr at this time period is directing the development of the T1-specific morphologies in the adult leg

(Fig. 4.7). Although not examined, I presume that Antp expression in all three pairs of the thoracic legs stays similar to each other directing the adult leg development into the default T2 pathway (Fig. 4.7).

Up-regulation of Scr in the T1 leg at the prepupal stage may modify the default pathway in at least three ways (Fig. 4.7C). First, the strong expression in the proximal ventral region of the future tibia likely promotes the development of the epiphysis. The epiphysis bud may be seen in the late W+2 leg, but is not conspicuously developed until the pupal stage. Secondly, in the distal part of the future tibia, Scr is suppressing the development of the tibial spurs that would form on the T2 tibia. It is around this time, the tibial spurs of the T2 and T3 legs begin to grow. Finally, the tarsal expression of Scr is most likely regulating the growth of this segment making it smaller than that of the T2 leg (Fig. 4.1C). Although the difference in the size of the tarsal segment between T1 and T2 are mostly due to the shorter Ta1 and Ta2 in the former, Scr expression was expressed along the length of the whole tarsus (Fig. 4.1E, 4.6G, H). This situation is in contrast to Ubx expression in the enlarged T3 tarsus in the orthopterans (Mahooz et al 2005). In the three orthopteran species examined, Ubx expression was restricted to the segments within the T3 tarsus, which differed in size from the corresponding segments in T2.

The up-regulation of Scr on the ventral side of the tibia is consistent with its role in directing the ventral-specific morphology of the T1 tibia. However, the anterior ventral up-regulation in the tarsus is not consistent with its proposed role in overall size regulation of this segment that includes the dorsal side. There are three possible explanations for this. First, the size could be controlled by the weak expression of Scr observed in the whole circumference of the tarsus (Fig. 4.7C). Secondly, the strong anterior ventral expression may be exerting a non-autonomous effect in the surrounding area. Stern (2003) showed that even though Ubx is only expressed in the posterior part of the developing adult femur in *Drosophila*, it has an effect on the growth of the entire segment. Finally, it is possible that I missed a time period during which Scr was being expressed at high level in the whole circumference of the tarsus. In general, my

observations are consistent with the idea that Scr does not control the development of segment-specific features until the initiation of metamorphosis.

Is Ubx acting in a similar manner?

Extrapolating from the current study of Scr expression during the ontogeny of the T1 leg, I can make some speculations about how Ubx might be expressed after the developmental stages observed by Zheng et al (1999). The last time point observed by Zheng et al. (1999) was 50% E when Ubx was expressed only in the posterior part of the T3 coxa (Zheng et al. 1999). This is different from Scr, which was already expressed weakly in the T1 leg just prior to this stage. Therefore, it is possible that Ubx may not be expressed at all in the T3 leg of the larva or may start to be expressed at a weak level at a slightly later stage. During metamorphosis, Ubx would most likely be expressed or up-regulated in the future femur and the proximal tarsus (Fig. 4.7), both of which possess T3-specific features. The T3 femur is significantly smaller in size as compared to the T2 counterpart and the T3 tibia has an additional pair of spurs on the proximal part of the segment. The tarsus may not up-regulate Ubx as its morphology is very similar to that of T2. Whether the up-regulation will be limited to the posterior part of the leg is difficult to predict (Fig. 4.7C is depicting this case).

Do the uniform larval leg morphologies result from the lack of Hox expression?

The legs of hatchlings in many hemimetabolous insects already show segment-specific features. During embryogenesis, Scr and Ubx are expressed in parts of their legs that show these features (Mahooz et al. 2004; Rogers et al. 1997; Hughes and Kaufman 2000) and are required to produce them (Hughes and Kaufman 2000). The larvae of Holometabola, by contrast, often possess a simpler set of thoracic legs that show uniform morphologies. Based on the Ubx expression data from the two

lepidopteran species (Zheng et al.1999; Warren et al. 1994) and their own analysis in the embryonic legs of the beetle and lacewing larvae, Mahooz et al (2005) proposed that the legs of these holometabolous larvae do not express Ubx until the adult leg development is initiated. Contrary to the Ubx expression, my results with Scr indicate that the larval legs of *Manduca* develop a uniform morphology *despite* the expression of this Hox gene. However, whether this low expression has any function will be hard to test. Even if expression were knocked out, I would not be able to cause homeosis since they all look the same to begin with! Indeed, RNAi knockdown of *Scr* in *Tribolium* produced no effect on the T1 leg morphology (Curtis et al. 2001).

One interesting natural experiment to observe the consequence of strong Scr expression would be to examine the expression of Scr in larvae with Tl legs that differ in morphology from T2 and T3. These types of larvae are rare, but one good candidate is the larva of the lobster moth *Stauropus fagi*. The larva has a rather typical T1 leg. But the T2 and T3 legs are narrow and about twice as long as the T1 leg. In this insect, one would expect Scr expression to be high in the T1 leg.

Significance of Scr expression in the embryonic and the larval legs

Scr had two phases of expression in the T1 leg during embryonic development. The early expression was not unique to the T1 leg, but was part of the weak ubiquitous expression throughout the embryo. This type of Scr expression is not previously reported in other insects and it is difficult to tell what the significance of this expression domain might be. The late phase of Scr expression in the T1 leg was segment-specific. The expression came on after the completion of morphological segmentation so this expression probably does not have role in morphological development of the leg.

One possible role of the weak Scr expression in the late embryonic leg may be retention of segmental identity from the early embryonic stage. Initiation and maintenance of Hox gene expression are regulated by two separate mechanisms (Simon 1995). Initiation of Hox gene expression is controlled by a set of transcription factors

that pattern the anterior-posterior axis in insect embryos. These proteins are encoded by the gap genes and segment polarity genes, and provide positional information along the A/P axis, directly activating and repressing the Hox genes in appropriate segments. These gene products, however, disappear relatively early during the embryogenesis (4 hrs AEL in *Drosophila*). For the rest of the life of the insects, the second mechanism maintains the activated and repressed states of appropriate Hox genes in cells. Sets of genes called *Polycomb-Group (PcG)* and *Trithorax-Group* (*trxG*) regulate this process. PcG proteins lock in the chromatin structure around Hox genes to a "closed" state to make them inaccessible to transcription machinery, while trxG proteins maintain it in an open state competent for transcription. It is thought that if a Hox gene is not activated at least once during embryogenesis, it will be locked in a closed state by PcG proteins (Mahmoudi and Verrijzer 2001; Poux et al. 1996). Thus, unless Scr is activated in the leg cells of *Manduca* during embryogenesis, they may not be able to turn it on at the time Scr is needed for the adult development in T1.

Although I could only speculate on the possible role of Scr expression in the larval leg, its spatial and temporal up-regulation during metamorphosis is consistent with their role in development of T1 specific-structures in some hemimetabolous insects and *Drosophila*. It would be interesting to examine whether Scr has similar temporal and spatial expression patterns during the ontogeny of other holometabolous insects. Results from such studies would confirm the proposal that the region-specific up-regulation of Scr during metamorphosis underlies the segmental differentiation of the thoracic legs in the adult holometabolous insects.

Acknowledgements

We would like to thank Paul Liu for generously providing the Scr antibody.

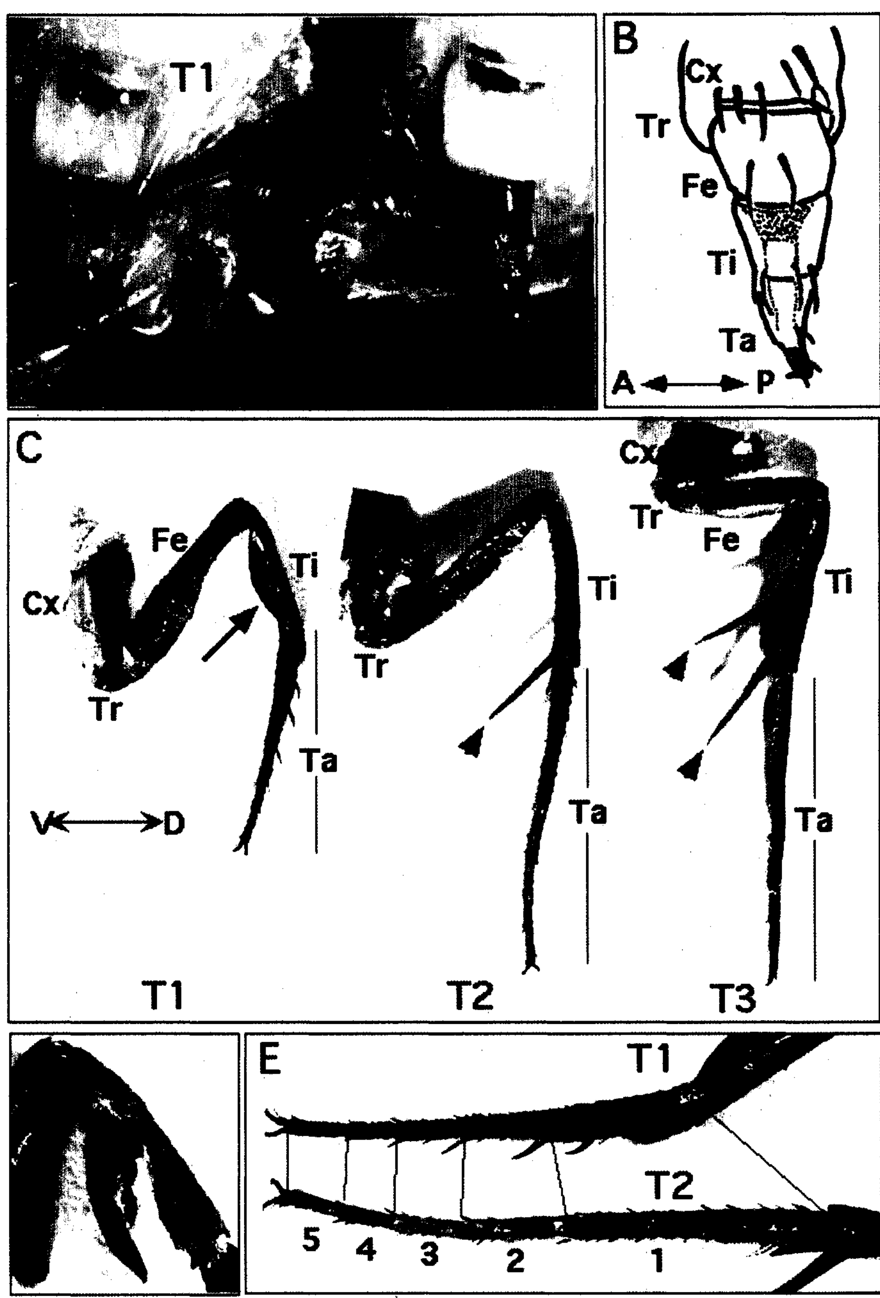


Figure 4.1. The thoracic legs of larval and adult *Manduca*. (A) Ventral view of the T1 and T2 legs in the V+0 larva. (B) Schematic drawing of the larval legs seen from the ventral side, showing the leg major bristles found in the legs on all the segments (red). A, anterior; P, posterior. (C) The adult legs. Femur lengths are similar between T1 and T2 legs, but shorter in T3. The tibia and tarsus are significantly shorter in T1 compared to the other legs. Epiphysis (arrow), an organ for cleaning antenna, is found on the ventral proximal part of the T1 tibia. The T2 tarsus has a pair of spurs at the ventral distal region (arrowhead). The T3 tibia has an additional pair of spurs in the medial ventral regions (arrowheads). V, ventral; D, dorsal. (D) Magnified view of the T1 epiphysis. Note that its length approaches that of the tibia. (E) Magnified view of the T1 and T2 tarsi. Difference in the tarsal length between T1 and T2 legs is mainly due to smaller tarsal segments 1 and 2 in T1. T1, first thoracic segment; T2, second thoracic segment; Cx, coxa; Tr, trochanter; Fe, femur; Ti, tibia; Ta, tarsus.

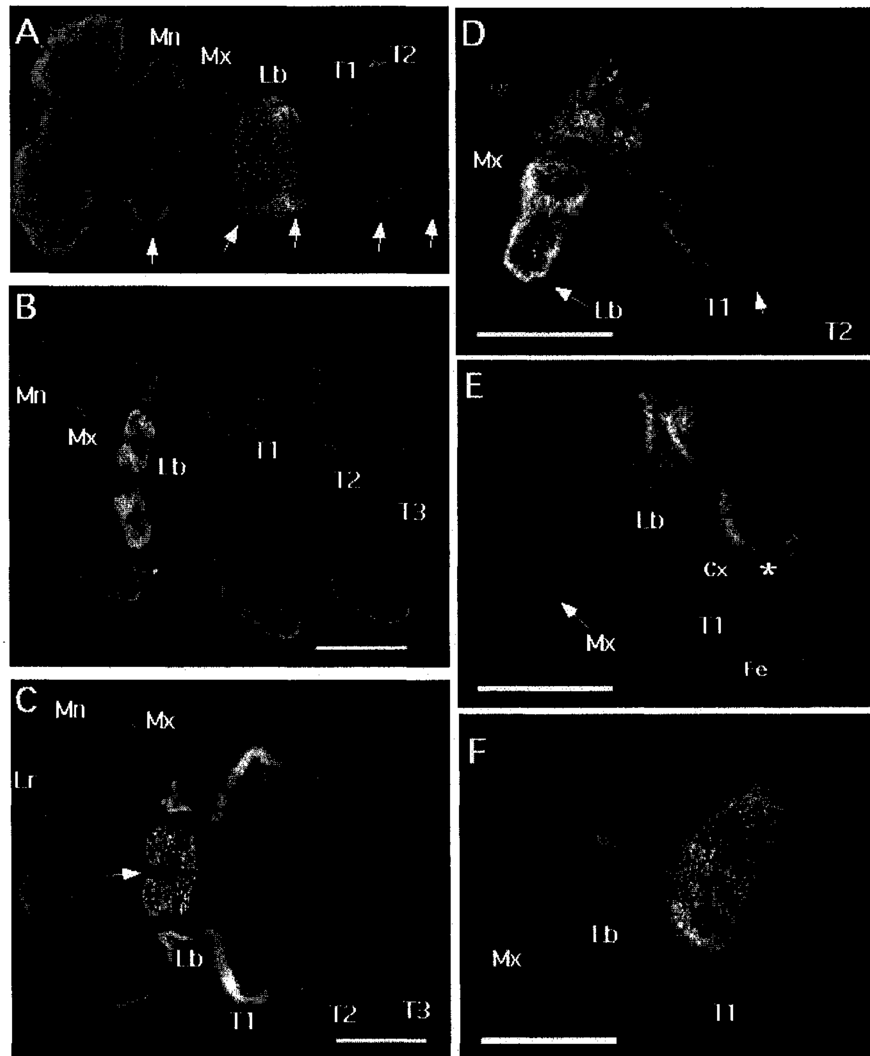


Figure 4.2. Scr expression in early and mid stage embryos. (A) ~14% E embryo. Ventral view. Strong expression of Scr is detected in the labial segment (Lb) including the nascent limb bud. Weak expression is seen in rest of the embryo. Arrows point to limb buds on each segment. (B,C) 36% E embryo. Ventral view. (B) Ventral focal plane showing appendages on the mandibular to T3 segment. Scr is detected intensely in the labial appendages (Lb). Mandible (Mn), maxilla (Mx) and thoracic legs do not show any expression. (C) More dorsal focal plane shows strong Scr expression in the body wall of the labial and T1 segment. The ganglion in the labial segment also expresses Scr at a high level (arrow). (D-F) 36% E embryo. Lateral view. (D) Medial view showing the labial segment (arrow) and the T1 body wall with strong Scr expression. Note that the posterior part of the T1 segment lacks the expression (arrowhead). (E) More lateral view showing Scr expression in the anterior coxa of T1 leg. Asterisk, expression in the mesodermal derivatives. (F) Lateral most view showing the Scr expression in the T1 body wall. Cx, coxa; Fe, femur; Lr, labrum. Anterior is to the left. Scale bar = 100 μm.

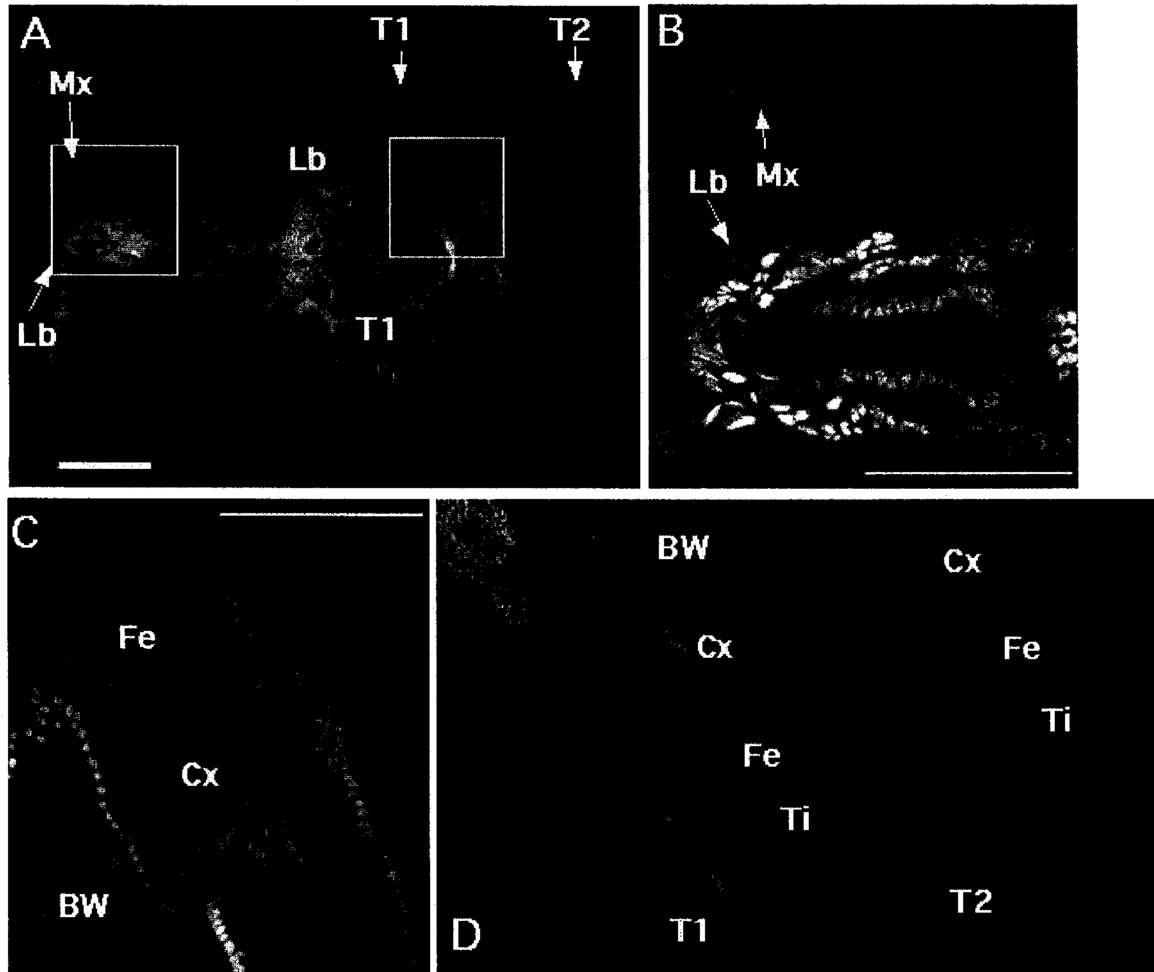


Figure 4.3. Scr expression in the leg during late embryogenesis. (A) 48% E. Ventral view. Labial appendages (Lb) have fused and projected forward at this stage. Boxed areas correspond to the enlarged views in (B) and (C). (B) Magnified section through the labial and maxillary appendages (Mx) in (A). (C) Magnified section through the T1 leg and body wall in (A). (D) Lateral view of T1 and T2 legs from the same stage. Cx, coxa; Fe, femur; Ti, tibia; Mx, maxilla; Lb, labium; BW, body wall. Scale bar= 100 μm.

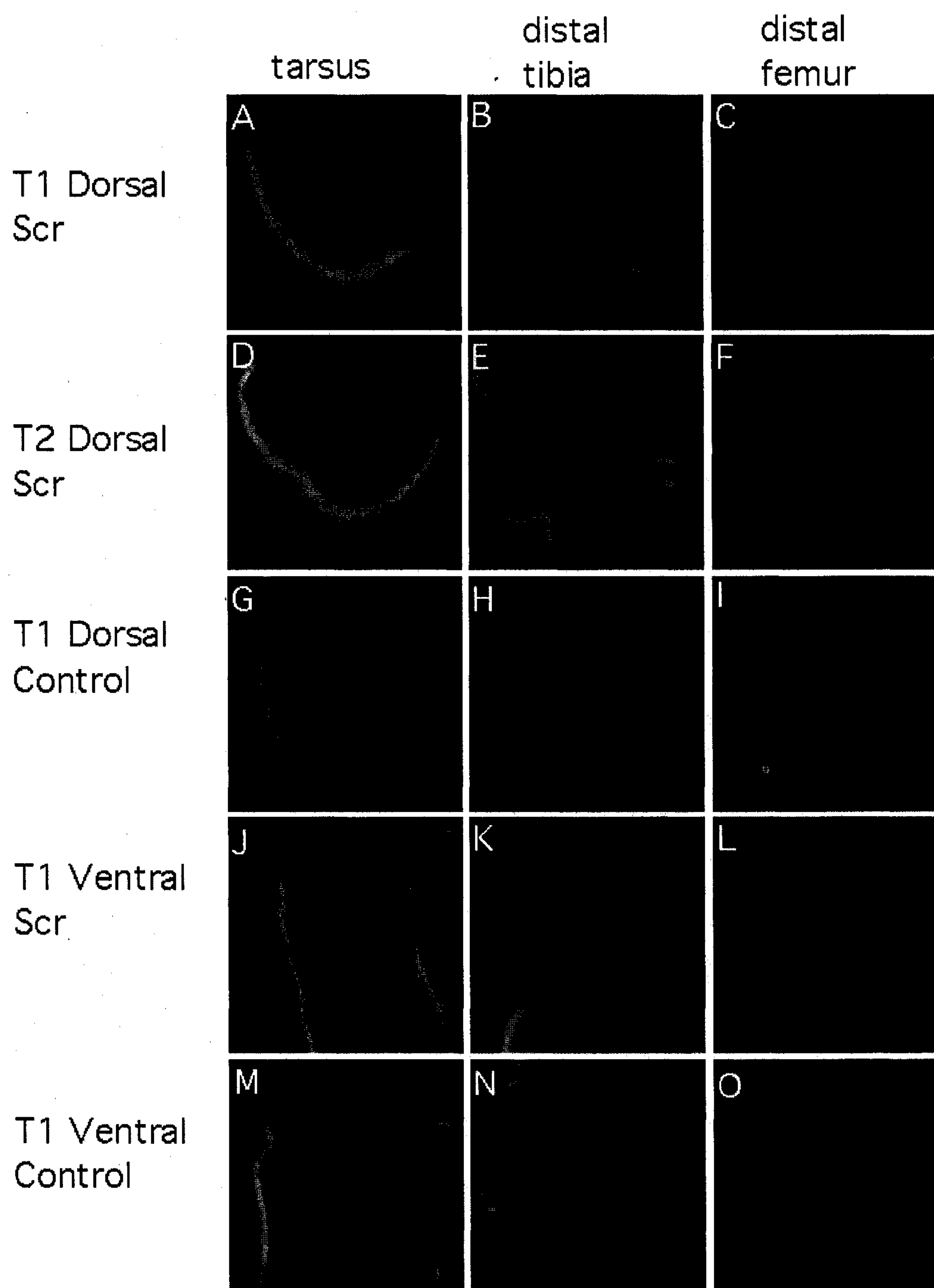


Figure 4.4. Scr expression in V< 2hr legs. (A-C) Dorsal T1. Clear Scr immunoreactivity is detected in the tarsus (A). The tibia shows weaker expression (B), whereas the femur only shows a low to background level of stain (C). Note there is no difference in expression level between the anterior and posterior sides (left and right). (D-F) Dorsal T2. Expression patterns are similar to those seen in T1. (G-I) No primary antibody control on the dorsal T1. No nuclear fluorescence is detected in the tarsus (G), tibia (H) and femur (I). (J-L) Ventral T1. Clear Scr immunoreactivity is observed in the tarsus. Moderate signals are detected in the tibia (K) and weaker signal in the femur (L). (M-O) No primary antibody control on the ventral T1.

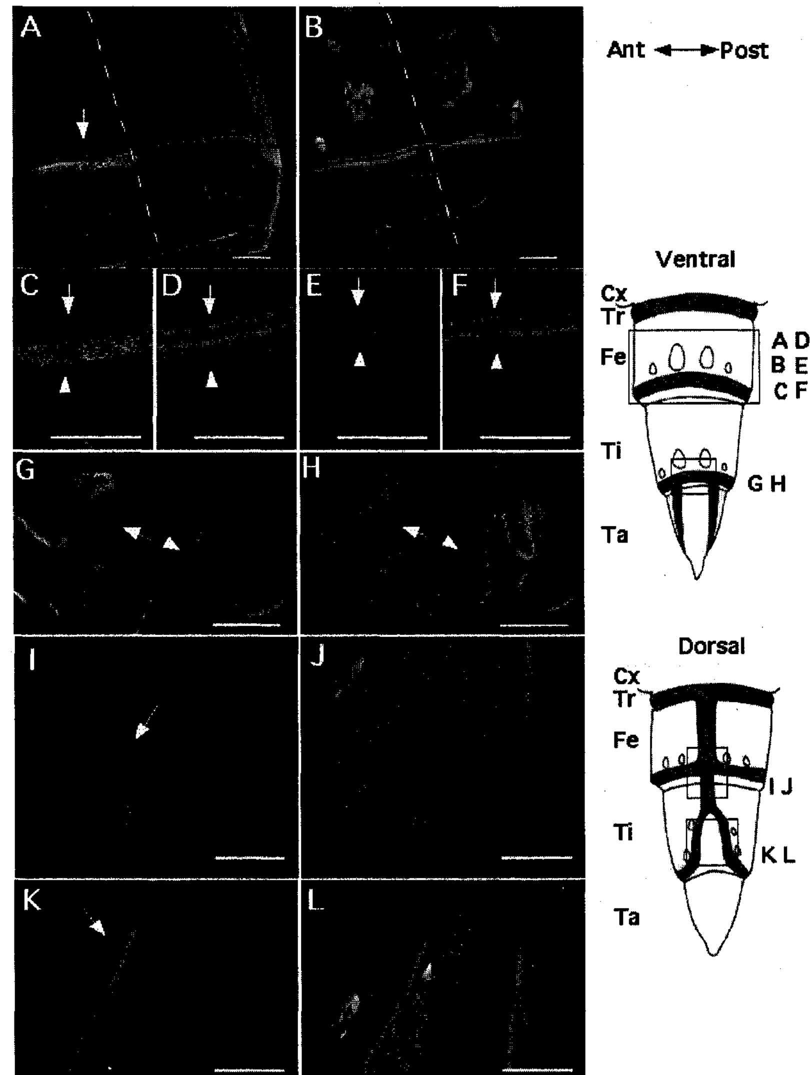


Figure 4.5. Scr is up-regulated in the developing primordia in the T1 femur and the tibia at V+ 72hr. Anterior is to the left in all the images. Diagrams on the right show the location of images in the whole leg. Shaded areas, location of the developing adult primordia. (A-F) Ventral femur. Dashed lines show the anterior-posterior border (A) Scr immunoreactivity. Expression is up-regulated on the anterior side of the primordium (left side). (B) Propidium iodide (PI) stain shows all of the nuclei in the leg epidermis. Large nuclei belong to the bristle cells. (C, D) Magnified view of the anterior part of the primordium in (A) and (B) respectively. Scr immunoreactivity (C) appears to be stronger on the distal side of the primordium (arrowhead) compared to the proximal side (arrow) (C). The PI stain shows no difference (D). (E, F) Magnified view of the posterior part of the primordium in (A) and (B) respectively. (E) Scr expression is significantly lower compared to the anterior part (C) and shows no difference between the distal (arrowhead) and the proximal (arrow) sides. (G, H) Primordium in the ventral tibia. Comparing Scr immunoreactivity (G) with PI staining (H), Scr expression is much stronger on the anterior side (arrow). (I, J) Primordium in the dorsal femur. Scr expression (I) is stronger in the distal anterior area (arrow) based on the comparison with the PI stain (J). (K, L) Primordium in the dorsal tibia. Comparison with the PI stain (L) shows the Scr expression (K) to be much stronger on the anterior side (arrow). Scale bar = 100 μm. The diagrams on the right show the locations of each panel within the leg. The areas shown in grey are the adult primordia.

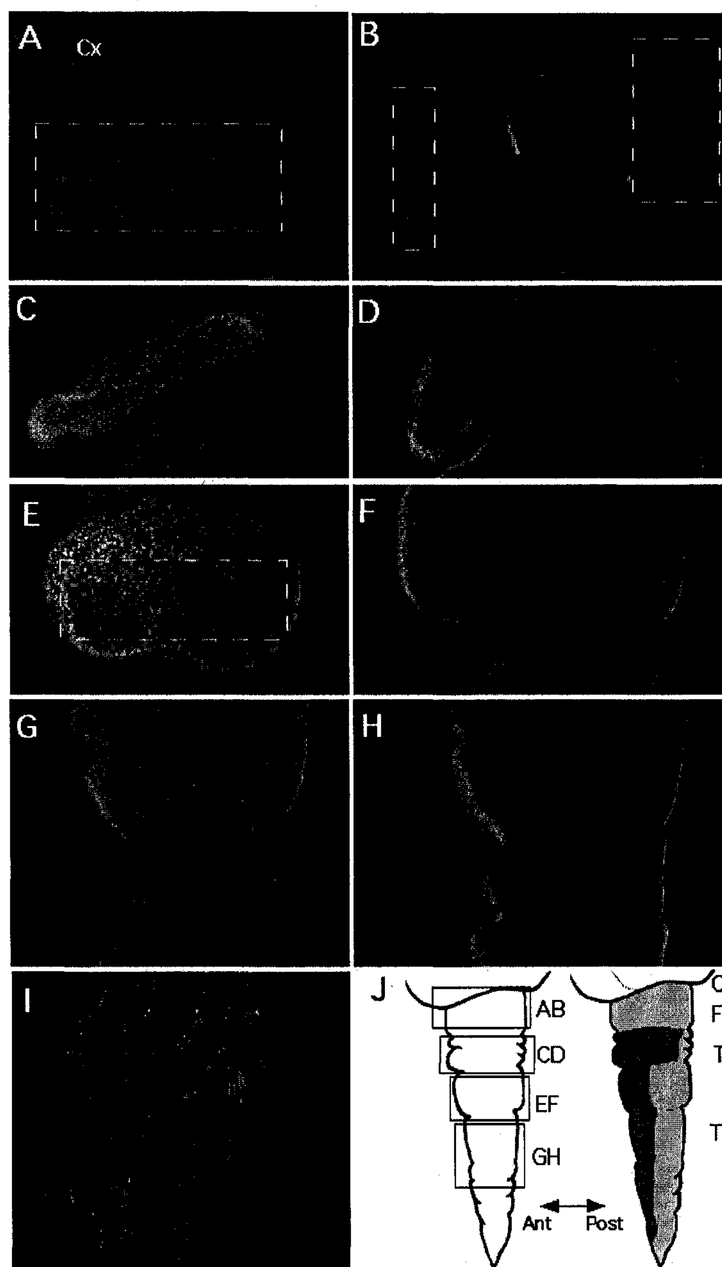


Figure 4.6. Scr expression in the mid W+2 leg. Anterior is to the left. (A-H) Ventral view of the mid W+2 T1 leg. (A, C, E, G) Confocal projection of ventral surface. (B, D, F, H) Single confocal sections of a more dorsal aspect. (A, B) Presumptive pupal femur. Weak immunoreactivity is detected throughout this area, but the area derived from the larval regions (inside the dotted lines) show stronger stain. The coxa (Cx) also shows clear immunoreactivity. (C, D) Scr is up-regulated on the whole ventral side of the presumptive pupal tibia (C). More dorsally, the expression is stronger on the anterior side (D). (E, F) Distal region of the presumptive pupal tibia. Area derived from the larval region is inside the dotted lines. Expression is up-regulated on the anterior side. (G, H) In the presumptive pupal tarsus, Scr is expressed at a high level on the anterior side. (I) Confocal section of T2 tibia and tarsal region. No immunoreactivity is detected in the epidermis, but there is staining inside the leg. This subepidermal stain was weaker in the T1 leg. (J) Diagrams showing the locations of each panel (left) and summarizing the expression patterns in the mid W2 T1 leg. Dotted boxes in (A), (B) and (E), the areas derived from the larval regions in the larval leg. Cx, coxa; Fe, femur; Ti, tibia; Ta, tarsus.

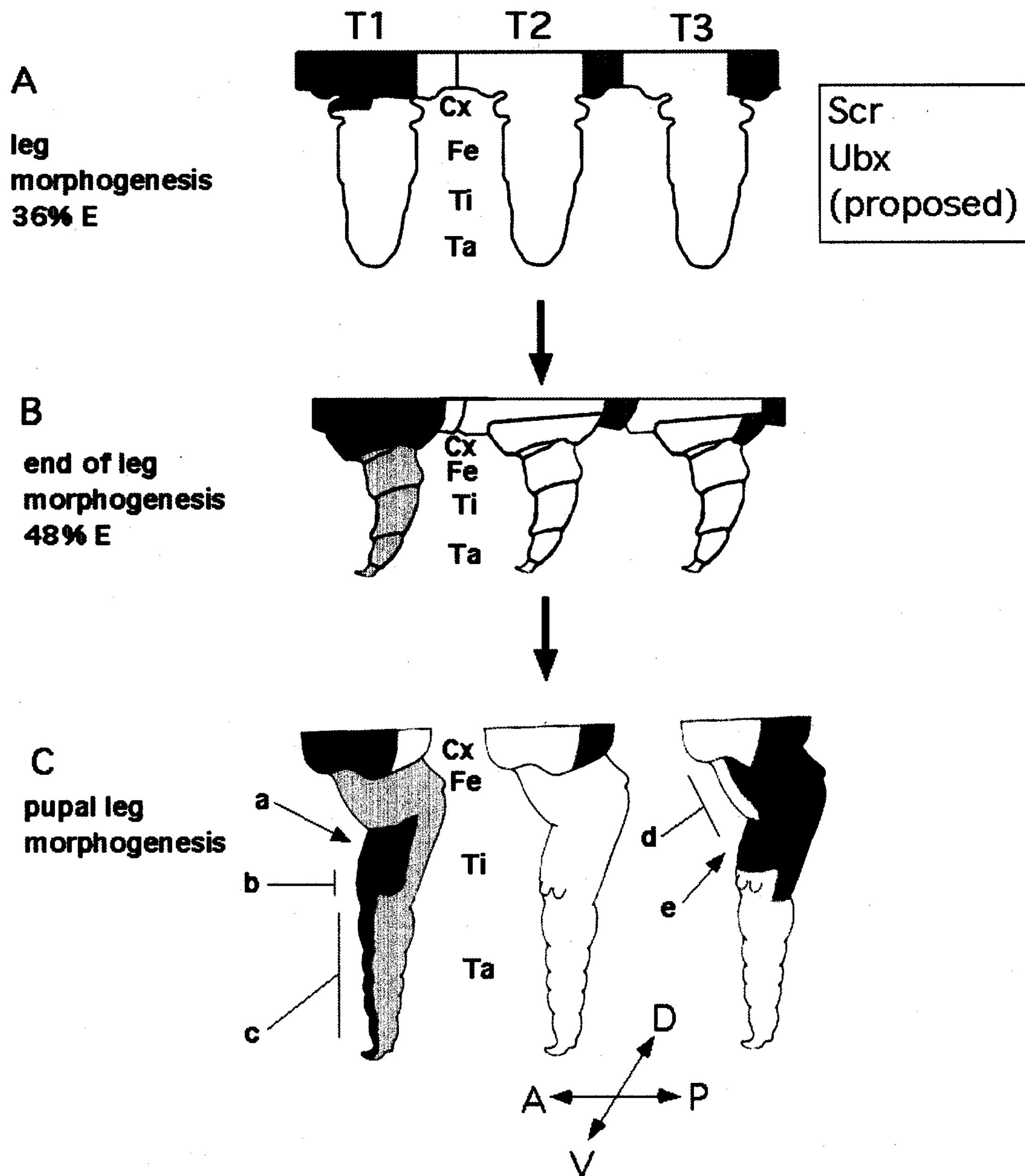


Figure 4.7. Model of segment specification of leg development by the thoracic Hox genes. The model is based on this study and Zheng et al (1999). Red, Scr; Green, Ubx; lighter colors represent weaker levels of expression. Antp is expressed in all three legs. (A) At the time of completion of the morphological segmentation (except the trochanter), Antp has been expressed in the leg. Scr is expressed in the anterior T1 coxa. (B) By the time of the shedding of the embryonic cuticle, Scr is weakly expressed in the T1 leg. Ubx expression appears in the posterior T3 coxa. (C) In W+2 legs, Scr is up-regulated in the ventral part of the T1 leg to promote the development of the epiphysis (a), suppress the spur formation (b) and regulate the growth of the tarsus (c). In the T3 leg, I speculate Ubx to be up-regulated in the femur to control its growth (d) and in the ventral tibia to promote the proximal pair of spurs. See text for more details. V, ventral; D, dorsal; A, anterior; P, posterior. Cx, coxa; Fe, femur; Ti, tibia; Ta, tarsus.

Chapter 5

Conclusion

During the evolution of the holometabolous insects, morphologically distinct larval stages were inserted into the more direct hemimetabolous life history. They have succeeded in accommodating this alteration without affecting the adult body form by inventing the pupal stage where most of the remodeling takes place. In this thesis, I have mainly dealt with two aspects of developmental processes underlying this remarkable life history, namely, the generation of a novel larval form and the delayed production of the adult body form. In this chapter, I will discuss these two issues in light of my findings in the *Manduca* leg and a few studies from other insects.

Developmental mechanisms underlying generation of larval forms

As mentioned in Chapter 1, the larval form is generally a result of three organismal-level phenomena. First, the appearance of the adult-specific organs is suppressed until the end of the larval life ["dissociation of developmental features" (Nagy and Grbic 1999)]. Second, for the organs functional in both the larval and the adult stages, the larval form is produced during embryogenesis and transformed into the adult form during metamorphosis. Finally, the larval-specific structures are produced during embryonic development or the larval stages and discarded at metamorphosis. The latter two are the primary processes by which the divergent larval forms are generated in Holometabola. Here I summarize some of the molecular and cellular mechanisms that underlie these developmental phenomena.

Interruption of the adult patterning program to produce simple larval forms in organs with biphasic development

The molecular mechanism responsible for patterning of the PD axis of the adult insect leg is largely conserved (Angelini and Kaufman 2005b; Fig. 3.2, 10). Data from studies of the adult leg patterning in the model holometabolan *Drosophila*, and the hemimetabolans *Oncopeltus* and crickets showed conservation of expression patterns of the PD genes, *Dll*, *dac*, *exd/hth/tsh*, *al* and *bab* (Angelini ant Kaufman 2004; Inoue et al. 2002; Erezyilmaz et al. 2004b). Of these, functions of *Dll* and *dac* have been tested in both *Drosophila* and *Oncopeltus* and shown to be conserved. In *Manduca*, I found that the expression patterns of these genes are also very similar in the developing pupal leg. This adds further support to the observation that the adult leg is produced by patterns of the PD gene expression highly conserved among insects that differ in their modes of adult leg development (Hemimetabola, Holometabola with larval legs and those without larval legs). The timing at which this pattern is established, though, varies among different groups. In Hemimetabola, in which the first instar nymph already has the adult leg form, the pattern is established during embryogenesis (Angelini and Kaufman 2004; Inoue et al. 2002; Erezyilmaz et al. 2004b). In flies, where the larval legs are virtually absent, the patterning occurs during the larval stages and is completed during the late last instar (Kojima 2004).

In *Manduca*, I found that the patterning process is initiated during embryogenesis as in the hemimetabolous insects because all of the PD genes examined are turned on (*Dll*, Exd, *dac*, Al and Bab). However, before the later steps are completed, the process is arrested in a immature state to give rise to the larval leg (Fig. 3.11). Only later during metamorphosis, is the conserved pattern of the PD gene expression established. Based on these data, I propose that the larval form of the *Manduca* leg is partly produced by truncating the conserved patterning cascade that would normally form an adult leg.

A possible arrest of the molecular patterning process in producing the larval form was also suggested by the study of Scr expression pattern during the segmental

differentiation of the thoracic legs (Chapter 4). In hemimetabolous insects, development of segment-specific features in the legs during embryogenesis are associated with the localized expression of Hox genes (Roger et al. 1997; Mahooz et al. 2004). Similarly, during the developmental periods in which the thoracic legs of the flies go through segment-specific development, Hox genes are expressed in the specific regions of the leg that go through such changes. In contrast to the adult legs in insects, the larval legs in Holometabola usually show no segmental differentiation. During larval leg development in *Manduca*, the Hox protein, Scr, was initially expressed in the nascent T1 leg bud as part of weak ubiquitous expression in the whole embryo. However, during most of the leg morphogenesis Scr was absent from the leg except for the coxa. After the completion of morphogenesis, a weak expression was observed throughout the T1 leg. A study of Ubx protein expression in the *Manduca* embryo (Zheng et al. 1999) reported that the protein was absent in the developing legs at least up to 50% E. During metamorphosis, though, I observed Scr to be up-regulated in the regions of the T1 leg that undergo segment-specific development (Fig. 4.6, 4.7). My observation is consistent with the proposal that the expression of Hox genes associated with the segment-specific development of the legs are suppressed temporarily during embryogenesis to give rise to the array of uniform larval legs.

These two cases of molecular patterning studies argue for the idea that the simplified larval leg forms are result of temporary arrest of the adult patterning system observed in the hemimetabolous embryos. It will be interesting to determine in the future whether other organs such as the mouthparts and antenna are produced by the same mechanism.

Division of organ primordia into the larval and adult populations

Another strategy for producing a larval-specific form of an organ is reported from comparative studies of development of the insect eyes and nervous systems (Liu

and Friedrich 2004; Truman 1996). In both cases, cells that produce the organs are divided into the larval and adult-specific populations.

In both the grasshopper *Schistocerca* and the beetle *Tribolium*, the eye fields are specified in the head during embryogenesis (Friedrich 2003). In the former, retinal differentiation sweeps across the eye field in an anterior-to-posterior direction resulting in an ordered array of photoreceptive cell units, the ommatidia (Liu and Friedrich 2004), and a third of the eye field has differentiated to form a small version of the compound eye by hatching. In subsequent nymphal molts, the eye increases in size anteriorly by further differentiation of the eye field (Friedrich 2003). In *Tribolium,* the embryonic eye field is partitioned into larval and adult regions (Friedrich 2003; Liu and Friedrich 2004). During embryogenesis retinal differentiation occurs only at the very posterior portion of the eye field. These differentiated cells are organized into a larval-specific visual organ, stemmata, consisting of roughly two dozen photoreceptors (Liu and Friedrich 2004). The rest of the eye field constitutes the adult region and remains undifferentiated until metamorphosis. At that time, it undergoes retinal differentiation and develops into a typical compound eye. Therefore, the eye field is divided spatially into the larval and the adult regions. The larval stemmata develop by repressing the progression of embryonic retinal differentiation after the formation of a small number of photoreceptors and organizing these in a larval-specific manner (Liu and Friedrich 2004). Thus, the stemmata can be considered homologous to the few rows of posterior ommatidia in the compound eyes of the hemimetabolous insects (Liu and Friedrich 2004).

An analogous strategy is used to make the central nervous system (CNS) in the holometabolous insects. During embryonic development, a full complement of the neuroblasts is generated and they produce neurons that differentiate into the larval-specific neurons. The majority of these neurons are eliminated during metamorphosis, while some of them remodel themselves to be part of the adult CNS. Most of the adult-specific neurons are produced from the second round of neuroblast division during the larval life and differentiate during adult development (Truman 1996; Truman and Riddiford 2002). In this case, the partitioning is temporal rather than spatial.

Molecular mechanisms underlying novel larval structures

Insect larvae possess larval-specific structures that have no adult counterparts and are discarded during metamorphosis. They are true evolutionary novelties in a sense that many of them have no homologous structures in the hemimetabolous ancestor. What are the molecular mechanisms underlying evolutionary origins of such structures? Would they be distinct from the ones that produce novelties in the adult form? To date, studies of molecular developmental mechanisms underlying origins of novelties in insects are limited to three structures, two of which are in the adult and one in the larva. In both adult structures examined, co-option and modification in expression of developmental transcription factors were involved (Hughes and Kaufman 2000; Moczek and Nagy 2005; Moczek et al. 2006). Hughes and Kaufman (2000) have examined the molecular changes underlying development of the sucking mouthpart in the hemipteran *Oncopeltus*. Although its mouthparts are not evolutionary novelty per se (because they were derived from the pre-existing chewing mouthparts), their morphology and function are radically different. Hughes and Kaufman (2000) found that the change in the expression pattern of the Hox gene *proboscipedia* and the resulting homeotic transformation of the maxilla into more mandible-like morphology underlie evolution and development of this novel mouthpart. In Holometabola, molecular studies of beetle horn development demonstrated that the PD genes responsible for the appendage patterning such as *Dll* and *al* were co-opted to make the horn (Moczek and Nagy 2005; Moczek et al. 2006).

The most illuminating studies of molecular mechanisms underlying development of larval-specific organs have focused on the abdominal prolegs in two orders of Holometabola. The prolegs are locomotory appendages present on the larval abdominal segments in several holometabolan orders. The abdominal segments of insects are normally prevented from making ventral appendages by the Hox genes *abdominal-A* and *Abdominal-B* (*abd-A* and *Abd-B*), which repress the limb-promoting gene *Dll*. However during the embryonic development of Lepidoptera, the abdominal

Hox genes are repressed in the ventral regions of the third through sixth and the last abdominal segments resulting in derepression of *Dll* (Warren et al. 1994; Zheng et al. 1999). Reactivation of *Dll* in these spots is thought to promote development of the prolegs (Warren et al. 1994; Zheng et al. 1999; Nagy and Grbic 1999). Therefore, both modification of Hox gene expression and co-option of *Dll* underlie development of the lepidopteran prolegs, presenting a situation similar to the two adult novelties discussed above. Nagy and Grbic (1999) further proposed that the prolegs are serially homologous to the thoracic legs, because they develop by reactivating the limb patterning network. However, my analysis of other components of the network, specifically, *Dll*, *dac*, *bab* and *al* (the latter two based on their protein expression patterns), showed that *Dll* was the only gene being deployed in the proleg development in *Manduca* (data not shown) and does not support this hypothesis.

Proleg development in the embryos of two sawfly species (Hymenopterans), which also possess the abdominal prolegs, has also been studied (Suzuki and Palopoli 2001). In contrast to the findings in the Lepidoptera, neither repression of the abdominal Hox genes nor expression of Dll protein in their abdominal prolegs was observed, suggesting that a different molecular mechanism may be responsible for their development. As there is no solid evidence for or against the homology of the prolegs in Lepidoptera and Hymenoptera, I cannot say which molecular changes were responsible for the evolutionary origin of the larval prolegs. If they evolved independently in each of these orders, different genetic mechanisms were used to evolve the structures with similar morphology and function (convergent evolution). On the other hand, if they evolved in the common ancestors of the two orders, then the developmental mechanism that was originally responsible for their evolution have been modified in one or both lineages.

How do you interrupt the adult patterning system in Manduca leg?

My findings suggest that an interruption of the PD patterning in the leg is partially responsible for producing the larval leg in *Manduca.* However we do not have a good idea about the mechanism that causes this interruption. One obvious candidate is juvenile hormone (JH), whose titer rises toward the end of larval leg morphogenesis. I hypothesized that this increase may arrest further progression of the patterning cascade. If this were true, I would expect that the removal of JH at this critical time would result in a larval leg that displayed pupal and/or adult patterning characteristics. When Bergot et al. (1981) applied an anti-JH agent fluoromevalonate (FMev) (Quistad et al. 1981) to *Manduca* eggs to prevent JH synthesis in the embryo, such leg abnormalities were not reported. I repeated this experiment focusing specifically on leg development and saw no effect on development of the legs (data not shown). I have also conducted a culture experiment in which the JH producing organ, corpora allata was removed by severing the head from the embryo. The segmentation of legs proceeded normally on the same time course as that of the controls (N= 24; data not shown).

The above experiments indicate that in *Manduca* and likely in Lepidoptera, JH apparently does not play a role in the arrest of the patterning process. Lepidopterans are relatively derived within the Holometabola so the possibility remains that in the ancestral Holometabola, the adult leg patterning system was sensitive to JH and could be suppressed. Loss of this suppression may have then occurred during the evolution of the Holometabola. A possible way to circumvent this problem is to conduct the analogous experiment in more basal holometabolous insects, such as snakeflies.

Delayed production of the adult form

Transformation of the larval organ form into the adult form requires a precise coordination of growth and patterning. Some issues involved in this process are: 1)

which cells in the larval organ to use for making the adult form; 2) resumption of the molecular patterning in a given cellular context; 3) patterned growth (morphogenetic growth) to make the adult form and building adult-specific structures and 4) the mechanism initiating the growth and patterning process. Here I address these issues in the context of my findings and what is known to date from other studies.

Relationship between the cells in the larval and the adult legs

My study in *Manduca* reported in this thesis and a study in *Tenebrio* adult leg development (Huet and Lenoir-Rousseaux 1976) show two ways in which the cells in the larval legs are deployed to make the adult legs. In *Manduca* legs, a subset of cells in the larval leg epidermis give rise to most of the adult leg, while a small population of cells outside the primordia makes a minor contribution. The reminder of the cells, which constitute a large part of the larval leg epidermis, are eliminated through apoptosis during the prepupal stage. All of the cells that comprise the adult leg epidermis are polymorphic in that they make the larval, pupal and adult cuticles. The adult primordia are located in the regions of the larval leg epidermis not associated with highly differentiated larval structures such as muscle attachment site and sensory bristles. This allows the animal to initiate adult morphogenesis early while the surrounding cells are still engaged in the larval leg function (Svacha 1992; Truman and Riddiford 2002).

An alternative and, most likely, more ancestral strategy is observed in the beetle *Tenebrio*. The beetle leg reportedly lacks special regions resembling the adult primordia and most, if not all, the larval epidermal cells appear to contribute to the adult leg (Huet and Lenoir-Rousseaux 1976; Truman and Riddiford 2002). In this case the cells may not be able to detach and proliferate to initiate the pupal leg morphogenesis until the locomotory and sensory functions of the larval leg are no longer necessary.

Resumption of the PD patterning process

The two ways of using the cells in the larval leg to make the adult form impose different conditions for the resumption of the patterning process. In *Manduca* since only a subset of the cells in the larval legs are used for the adult leg, some cells shift their segmental identities. Some cells in the primordia in the larval femur and tibia end up becoming part of the adult tibia and tarsus respectively. These changes are also accompanied by changes in their molecular identities characterized by the expression of specific PD genes. In beetles, no molecular patterning of the leg during the adult leg development has been examined. However, based on the observation that all the epidermal cells in the larval leg contribute to the pupal leg, a change in the segmental identities would not be expected to be as extensive as that seen in *Manduca*.

In addition to the above changes, the overall pattern of the PD gene expression is refined into those of the conserved adult pattern during *Manduca* metamorphosis. By W+2, the Bab expression domain in the tarsus resolves into four stripes of strong expression flanked by regions of weaker expression. Also, *dac* and Bab, whose expression domains are mutually exclusive in the larval leg, become co-expressed in the proximal pupal tarsus.

Changes in allometric growth

The leg grows about 7 times in length during metamorphosis while the length of the body remains about the same. In addition to this sheer increase in size, the changes in shapes of the leg segments result from differential growth along the three axes in each leg segment. The potential molecular candidates involved in this process are the genes affecting leg growth such as the joint patterning genes, *Notch*, *Delta*, *Serrate* and the morphogens Decapentaplegic, Wingless and Hedgehog. The Hox gene *Ubx* was also shown to be involved in controlling segment-specific growth both cell autonomously and non-autonomously in the T3 leg of *Drosophila* (Stern 2003) during

adult leg development. Consistent with this finding, in *Manduca*, *Scr* was expressed in the segments of the developing adult T1 leg that differ in size from the T2 and T3 legs, thereby, likely affecting their growth. More recently, the transcription factor *Broad* was shown to be necessary for changes in organ shape through nymphal instars in the hemimetabolous bug *Oncopeltus* (Erezyilmaz et al 2006).

Integration of multiple molecular inputs during metamorphosis

The adult legs of *Manduca* feature structures unique to the adult stage such as the tibial spurs and the epiphysis. Why do these structures develop in the adult, but not in the larva? Most likely, their development is initiated by a few genes, which do not get expressed or up-regulated at the future location of these organs until metamorphosis. These genes could encode transcription factors such as *Dll*, which is deployed repeatedly in development of novel structures such as the beetle horn (Moczek and Nagy 2005), the larval tail horn of *Manduca* (Zheng et al. 1999) and vast array of appendages in the metazoans (Panganiban et al. 1997). Positional information from the new PD gene expression patterns (along with already existing dorsoventral and anterior-posterior signals), segmental identity from Hox genes and temporary cues from hormonal titers are likely be integrated on an enhancer in *cis*-regulatory regions of these genes and regulate their expression.

What initiates the resumption of the adult patterning and growth?

As described above, the morphogenesis of the pupal leg involves both changes in the transcription of the PD genes and tissue growth. The results from Chapter 3 and Truman et al. (2006) suggest that changes in transcriptional activities of some genes precede the initiation of cell proliferation. The mRNA of the PD gene *Dll* (Fig. 3.10) and the transcription factor *br* (Truman et al. 2006) was detected in the leg before the

onset of the marker for cell proliferation. Based on the order of these events, it is possible that the former events initiate the latter one. However, what needs to be determined is the factor that triggers this early transcription (and maybe separately the tissue growth) at the beginning of the last instar. Recently, Truman et al. (2006) have shown that JH has an ability to regulate some aspect of these early events in *Manduca.* When the last instar larva was starved, neither the growth of the primordia nor the transcription of *br* was initiated (Truman et al. 2006). However, if the source of JH was removed, then both *br* transcription and growth occurred, although the resulting discs were smaller in size (Truman et al. 2006). Similarly, when I starved early last instar larvae, *Dll* transcription was not observed as in normally fed larva. When the source of JH was removed, *Dll* expression occurred in the starved larvae (personal observation). These results indicate JH has a role in either directly suppressing the transcription of *Dll* and *br* or a factor upstream of their transcription. It has yet to be determined whether there is a molecular event preceding *Dll* and *br* transcription.

Generation of two body forms in a single ontogeny is an incredibly complex task. The studies discussed in this chapter and my thesis work offer a glimpse into a number of developmental processes involved and various strategies by which the holometabolous insects achieve this feat. On the surface, the evolution of holometabolous life history appears to have occurred by a simple insertion of a pupal stage. However, in reality, most, if not all, of the developmental processes discussed above had to be put in place in order for this life history to evolve. Mechanistic investigation of many of these processes have yet to be undertaken and will be a challenge, because the insects in which they are most prominent are not developed as an experimental system, and as such, do not have the sophisticated tools of developmental genetics. Further understanding of the way in which the holometabolous insects create two disparate body forms and how it evolved will require investigations of all of these processes and experiments using insects in different phylogenetic positions.

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