**LETTER**

**Larval Legs of Mulberry Silkworm Bombyx mori Are Prototypes for the Adult Legs**

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Summary: Morphological diversity of leg appendages is one of the hallmarks of developmental evolution. Limbs in insects may develop either from their embryonic prototyes or from imaginal discs harbored inside the larva. Bombyx mori (B. mori), a Lepidopteran insect, develops adult wings from larval wing imaginal discs. However, it has been debated whether the adult legs of B. mori arise from imaginal discs or from the larval legs. Here we addressed how the larval legs relate to their adult counterparts. We present the morphological landmarks during early leg development. We used expression of developmental genes like Distalless and extradenticle to mark leg primordia. Finally, we employed classical excision approach to develop a fate map of the adult leg. Excision and ablation of thoracic legs along proximodistal axis at various times during larval development resulted in the loss of corresponding adult leg segments. Our data suggest that B. mori legs develop from larval appendages rather than leg imaginal discs. gene his 45:169–176, 2007. © 2007 Wiley-Liss, Inc.

Key words: Bombyx mori; silkworm; limb development; lepidoptera; imaginal disc; drosophila

**INTRODUCTION**

During evolution there has been a trend towards the divergence of the number, morphology, and function of limbs within different taxa (Carroll, 2000; Carroll et al., 2001; Jockusch et al., 2000; Panganiban et al., 1997; Weatherbee and Carroll, 1999). Studying the mechanisms of development of these homologous structures is crucial in understanding the evolution of animal body plans (Carroll et al., 2001; Jockusch et al., 2000; Jockusch et al., 2004; Niwa et al., 2000; Weatherbee and Carroll, 1999). The adult body pattern in insects may develop either from juvenile instars that resemble the adult or from imaginal discs (Carroll et al., 2001; Friedrich and Benzer, 2000; Truman and Riddiford, 1999, 2002). Imaginal discs are sacs of epidermal cells specified during embryonic development that proliferate during larval development and differentiate during pupal metamorphosis to give rise to adult derivatives (Cohen, 1993).

The mulberry silkworm Bombyx mori (B. mori) is a Lepidopteran insect (Gopinathan et al., 1997; Tazima, 1964; ). Lepidoptera are one of the most closely related insect orders to Diptera (e.g., Drosophila melanogaster) (Common, 1975; Regier et al., 1995). In Drosophila, all adult appendages develop from imaginal discs. B. mori larva has three pairs of thoracic legs and five pairs of abdominal legs. Abdominal legs are lost during larval to pupal metamorphosis (Gopinathan et al., 1997; Suzuki and Palopoli, 2001; Ueno et al., 1995; ). It has been debated for a long time whether the adult legs of the lepidopteran insects arise from imaginal discs or from larval legs (Kim, 1959; Svacha, 1992; Tanaka and Truman, 2005). Kellog and Bodenstein suggested that the larval legs are completely lost during metamorphosis and adult legs develop from imaginal discs. However, others suggested that adult legs in the lepidopteran moths, Galleria mellonella and Manduca sexta, do not develop from imaginal discs but from the tissue present within the larval legs (Kim, 1959; Svacha, 1962; Tanaka and Truman, 2005). Here we addressed the question of how do the larval leg appendages of B. mori relate to their adult counterparts.

**RESULTS AND DISCUSSION**

First, using scanning electron microscopy (SEM), we studied the morphology of the developing legs from embryo to adult. In B. mori, embryonic development spans 10 days. At 40 h after egg laying (AEL), three pairs of ectodermal evaginations originate ventrally on the...
pro-, meso-, and meta-thoracic segments (data not shown). These evaginations develop into bilaterally arranged tubular leg projections along the antero-posterior axis of the embryo at three days AEL (Fig. 1a,b). The abdominal legs of *B. mori* are small nonsegmented protrusions on the ventral surface of the larval abdominal epithelium (Fig. 1c). The embryonic thoracic legs have three segments (Fig. 1d). The segmented pattern further evolves to form the larval leg with five segments, where a claw forms the distal fifth segment (Fig. 1e). This segmented organization is maintained in the pupal (Fig. 1f) and the adult legs (Fig. 1g). The characteristic multijointed silkmoth leg comprises coxa, attached to the body wall, followed by the trochanter, the femur, the tibia, and five tarsal segments ending in a pair of claws (Fig. 1g). Although it is likely that cell death is induced to edit cells during metamorphosis to form adult legs, the morphology of the larval/pupal leg suggests that not all cells may be replaced during metamorphosis to form adult legs (Fig. 1e,f).

We used expression of functionally conserved genes *Distalless (Dll)* and *extradenticle (exd)* to follow the development of legs during embryogenesis (Beldade and Brakefield, 2002; Jockusch et al., 2000; Jockusch et al., 2004; Panganiban et al., 1995; Palpoli and Patel, 1998; Suzuki and Palopoli, 2001). *Dll* is expressed in ectodermal evaginations on all segments in a two-days-old embryo (Fig. 2a). This pattern evolves and is refined to the three thoracic-legs and five abdominal-legs, and the developing head segments in a five-days-old embryo (Fig. 2b). *Dll* expression remains unaltered up to 7.5 days of embryogenesis (Fig. 2c). *Exd* is expressed in the proximal cells of the legs. *Exd* is expressed strongly in both abdominal and thoracic legs of the larvae (Fig. 2d). The *Dll and Exd* could not be traced in subsequent embryonic stages because the resolution of staining was compromised due to strong autofluorescence, thickness, and opacity of the embryonic tissue.

**FIG. 1.** Leg prototypes are present during embryogenesis. Scanning Electron Micrographs (SEM) showing (a–c) 2 day old *B. mori* embryo showing bilaterally arranged (b) thoracic legs (arrowheads mark thoracic legs, arrow marks the longitudinal groove), (c) abdominal legs (arrows) on the ventral side of the embryo along the antero-posterior axis, (d) Magnified view showing three-segmented structure of the embryonic thoracic leg (I-III). (e) Fifth instar larval leg with five segments, where the fifth or distal most segment ends in a claw (segment V). (f) The pupal thoracic leg retains a well developed leg like structure. (g) The multi-segmented leg of adult (moth) showing: Coxa (Co), Trochanter (Tr), Femur (Fe), Tibia (Ti), Tarsi (Ta) and Claw (Cl). (TL 1–3: Thoracic Leg 1–3, AL3–5: abdominal leg 3–5).

**FIG. 2.** *Dll* and *Exd* mark leg primordia in the embryo. (a) Early expression of *Dll* is observed in all the developing segments of the embryo. (b) Expression evolves during later stages of development, which marks the progenitors of thoracic legs (tl), abdominal legs (al), caudal leg (cl) and the mouthparts in embryo at late fifth days AEL. (c) *Dll* expression does not evolve in later stages of embryonic development and remains confined to thoracic- (tl), abdominal- (al) and caudal- (cl) legs. (d) *Exd* expression marks both abdominal and thoracic legs of the embryo on early fifth day AEL. Dotted line marks the outline of the body.
Dissection of silkworm larvae during various stages of larval development readily revealed the wing imaginal discs (see Fig. 3), but not the leg imaginal discs. Therefore, we employed the classical approach of surgical excision or perturbation to ask whether the adult legs develop from larval legs (Fig. 4a). (a) in-situ perturbation: The pro-, meso-, and meta-thoracic legs were perturbed on the right side in the developing first- to fifth-instar larvae (details in Methods). This approach has some advantages: (i) Contra-lateral legs serve as controls and (ii) perturbing cells in situ allows studying the development and differentiation of cells within their normal segmental milieu.

Following perturbation, the pharate adults were recovered at a reasonable frequency of about 40–50%. These perturbations resulted in a range of defects in terms of segment number and size of the thoracic legs. Adult moths emerged with shorter and thicker legs although the number of leg-segments was not affected (Fig. 4b, Table 1). Rarely, some tarsal segments were either lost or fused (data not shown). When the perturbations were performed in medial leg segments, the adult thoracic leg showed shortening of the tarsal segments and formation of extra claws in 2 out of the 19 experimental moths (Fig. 4c, Table 1). A higher magnification of this region clearly showed that an extra claw is formed in the leg although the tarsal segments were fused or lost (inset Fig. 4c, arrowheads). It suggests that the cells within the larval legs contribute to the formation of the adult legs. However, if a subset of cells was required as seen in the tobacco hornworm moth, Manduca sexta (Tanaka and Truman, 2005) or cells from all larval segments were required, remained unclear. To address this issue, we designed experiments in which the larval leg was excised and its developmental fate was observed in the adult moth.

(b) Segmental Excisions: We carried excisions in three different domains (details in Methods). In distal excisions, the Segment V (claw) or Segments IV and V (tarsal segments and claw) of larval legs were excised (Fig. 1c). This resulted in adult thoracic legs lacking corresponding distal claw or claw along with tarsal segments (Fig. 3d, Table 1). The coxa, trochanter, femur, and tibia were normal, but instead of the normal complement of tarsal segments, the excised legs showed black scar tissue at the point of excision (Fig. 4d, arrow). The contra-lateral control legs were normal (Table 1). Another common phenotype of distal excision (10/21) was loss of distal tarsal segments and claw (Table 1). The distal tip of the tibia showed a black scar tissue and thickening at the point of excision (Fig. 4e, 4e'). It also suggests that these legs failed to regenerate the distal patterning elements.

The medial category involved excision at the Segment III leading to loss of Segment III, IV, and V of the larval leg (Figs. 1c, 5a). Such excisions generated severely reduced adult thoracic legs with a thicker coxa and elongated femur that lacked tibia (III), tarsal segments (IV), and the claws (V) (Fig. 4f, arrow, Table 1). The proximal excisions included removal of Segment II, III, IV, and V of the larval leg (Figs. 1c, 5a). Most of the larvae died 48 h after excision because of excessive loss of haemolymph only a few (4/46), survived to adulthood and showed adult legs with only coxa attached to the body wall. All other distal segments of the adult legs were missing. There was a scar tissue at the distal tip of these experimental thoracic legs marking the point of excision (Fig. 4g, Table 1). We also excised the entire...
Thoracic legs lack regeneration ability as early as second instar of larval development. (a) Segmental organization of the thoracic legs of larva and adult, and the two different approaches: (i) in situ perturbation, which involves teasing of the few cells of the larval leg tissue, (ii) segmental excision of larval leg at different lengths along the proximo-distal axis. (b) In situ perturbation in the second instar larval leg resulted in shortening of the thoracic leg of adult moth. The coxa and trochanter appear to be thicker in comparison to their wild-type counterparts while the tarsal segments become shorter due to thickening. (c) The presence of extra claws in the adult leg was due to in situ damage in the segment IV and V of the larval leg. (c') Inset shows the magnified view of tarsal region where an extra claw is formed (arrowheads). (d) Excision of distal segments IV (tarsi) and V (claw) of larval leg resulted in adult leg devoid of tarsal segments and claw (arrow). (e) Excision of the segment IV of larval leg generated adult leg with loss of claw and tarsal segments. (e') In these legs distal tibial segment ended in a thickened structure. (f) Excision of larval leg at segment III resulted in the formation of the coxa and elongated trochanter (arrow). (g) Proximal excision of larval leg including segment II, III, IV generated adult leg with only the coxa attached to the body wall. The point of excision is marked by black scar tissue (arrow). (CH: caudal horn).
larval legs near the body wall removing Segments I–V. These excisions were fatal to the larvae as 99% of the larvae died within 24 h after excision possibly because of excessive loss of haemolymph. In case of excision of entire larval leg in fourth- and fifth-instar larvae, some early pupae (3/79) were recovered, which were completely lacking the experimental leg whereas the contralateral control legs were perfectly normal (Table 1). These animals died as early pupae.

Taken together, excisions in larval leg directly corresponded to loss of pattern of the adult leg (Figs. 4f, 5a). It suggests that B. mori adult legs are generated from their larval counterparts (Fig. 5). This effect was seen as early as second instar of larval development. The high mortality rates did not permit fate mapping during first-instar larva. However, the development of multi-segmented thoracic legs at 40 h AEL raised the possibility that adult leg prototype is present as early as embryogenesis (Fig. 1). In-situ perturbation of the larval leg resulted in the development of abnormally patterned adult legs suggesting that the cell types responsible for forming the adult leg may be apposed to the larval leg cuticle.

Table 1

<table>
<thead>
<tr>
<th>Experimental strategy</th>
<th>Phenotypes</th>
<th>Controls (Contra-lateral legs of same adult moth)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perturbation</td>
<td>Shortening and thickening of adult moth legs (22/29)</td>
<td>No effect (22/29) 7a</td>
</tr>
<tr>
<td></td>
<td>Splitting of leg in tarsal region (2/19)</td>
<td>No effect 19/19</td>
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<tr>
<td>Excision/Segmental perturbation in larval leg (Fig. 2e)</td>
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<td>Distal:</td>
<td></td>
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<tr>
<td>Removal of Segment V</td>
<td>Adult moth legs lacking the distal segments</td>
<td>No effect</td>
</tr>
<tr>
<td>Segment IV + V</td>
<td>Claw (14/24)</td>
<td>(14/24) 10a</td>
</tr>
<tr>
<td></td>
<td>Claw + Tarsi (10/21)</td>
<td>(10/21) 11a</td>
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<tr>
<td>Medial:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Removal of Segment III + IV + V</td>
<td>Adult moth legs lacking tibia (11/17)</td>
<td>No effect 11/17 (6a)</td>
</tr>
<tr>
<td>Proximal:</td>
<td></td>
<td></td>
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<tr>
<td>Removal of Segment II + III + IV + V</td>
<td>Adult moth legs growing a small structure near the body wall. This structure can be coxa (4/46)</td>
<td>No effect (4/46) 42a</td>
</tr>
<tr>
<td>Segment I + II + III + IV + V</td>
<td>Larvae died due to excessive loss of haemolymph (0/79)</td>
<td>(0/79) 79a</td>
</tr>
</tbody>
</table>

7a Represents the number of experimental larvae that failed to form pupae. Since the contra-lateral side of each experimental moth served as controls, due to early lethality, the control phenotypes could not be seen in these moths.

FIG. 5. Patterning of adult appendages in B. mori. (a) A fate map of silkworm thoracic leg. Our results demonstrate that larval leg segments from I to V give rise to the adult leg coxa, femur, trochanter, tibia, tarsi, and claw, respectively. (b) The thoracic legs develop from their larval prototypes whereas wing develops from wing imaginal disc like group of cells present in meso- and meta-thoracic segments, which give rise to the fore- and the hind wing respectively. Interestingly, thoracic leg primordia are present even in the embryonic stages of development. (H: head, MP: mouthparts; tl: thoracic leg, AL: abdominal leg, cl: caudal leg, T1–3: thoracic segments, A1–11: abdominal segments FWD: fore wing imaginal disc, HWD: hind wing imaginal disc AN: antenna, TL 1–3: adult thoracic legs, FW: fore wing, HW: hind wing).

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and this continuity was maintained in the cell types constituting the larval and adult legs. Our results also validate the studies on leg development of Manduca sexta (Tanaka and Truman, 2005). They showed that the simple thoracic legs of larva are remodeled into adult multi-segmented leg. Majority of adult leg epidermis develops from small group of epidermal cells located in specific regions of the larval leg. During metamorphosis these epidermal cells undergo rapid proliferation to give rise to adult leg structures. The results from our segmental excision approach suggested the possibility that similar developmental mechanisms may be involved in B. mori leg development.

Growth regulation is seen in many epithelia like the Drosophila imaginal discs or cockroach legs, which when cultured after fragmentation regenerate or duplicate the lost portions by intercalation of distal patterning elements (Anderson and French, 1985; Bryant, 1975; French et al., 1976). The lepidopteran models have exhibited variable response to excision (French, 1997; Kellog, 1904; Nijhout, 1991; Toussaint and French, 1988). In wings of B. annanyana, transplantation or epidermal damage revealed the capacity of cells to form ectopic eyespots (French, 1997; French and Brakefield 1995; Nijhout, 1991; Toussaint and French, 1988). Little or no regeneration was seen in cauterized wing discs of P. coenia, Philosomia cynthia ricini, Papilio machaon, and B. mori (Kango-Singh et al., 2001; Nijhout and Gru-nett, 1988). In B. mori, our results suggest that there is a strong possibility that larval legs lack regeneration ability as excision in larval leg resulted in loss of corresponding structure in the adult leg. However, absence of regeneration itself is not sufficient enough reason to conclude that the silkworms lack leg imaginal discs because the regenerative capability can be independent of the nature of precursor cells (imaginal discs or imaginal cells) that form the adult leg.

Kellog carried out excision experiments in B. mori legs (strain not known) and showed two sets of observations: (a) There was no regeneration observed when caudal horn or the entire thoracic leg of larva was excised. The caudal horn is a pointed non-segmented but movable process projecting upward from the dorsal surface of the eighth abdominal segment (Fig. 4a). (b) In only a few cases regeneration is observed when a part of the thoracic leg was excised. He suggested that penetrance in this phenotype is due to the fact that regeneration as a trait is probably lost in domesticated silkworm because of natural selection (Kellog, 1904). The silkworm has been domesticated for about 4,500 years. Therefore, the basis for maintaining the advantageous capacity for regeneration is not absolutely essential (Kellog, 1904). However, it seems highly unlikely that a deeply rooted mechanism such as regenerative capacity would be completely eliminated due to artificial selection. Our observation of lack of regeneration in Pure Mysore strain of B. mori raises another interesting notion that the larval legs have attained a state where individual centers/leg segments give rise to specific parts of adult legs and loss in a particular segment cannot be compensated by regeneration. However, it is possible that the embryonic leg primordium (see Fig. 1) may have the capability of regeneration.

Despite the similarity in organization of adult legs in Drosophila and B. mori, there are significant deviations in the mechanism of leg development. In Drosophila, all limbs generate form imaginal discs (Cohen, 1993). However, silkworm develops adult wing appendage from wing-imaginal disc (Fig. 3) and adult legs from their larval legs (Fig. 5). This makes silkworm as one of the ideal models to study patterning mechanisms as it may serve to mark the transition from development of limbs from larval prototypes to imaginal primordia. Furthermore, the B. mori genome is sequenced (Mita et al., 2004), and mutations in various patterning genes are available thus making it amenable to further investigate the molecular mechanisms controlling limb patterning.

**MATERIALS AND METHODS**

We used a nondiapausing multivoltine strain of B. mori, Pure Mysore to facilitate the staging of embryos. Egg collection and dechorionation were performed as described earlier (Singh and Gopinathan, 1997).

**SEM**

Dechorionated eggs from different developmental stages and dissected larval- and adult tissues were fixed overnight at 4°C in 5% glutaraldehyde in 100 mM Phosphate buffer, pH 6.8. The specimens were treated with a 1:1 mixture of 4% glutaraldehyde and 2% osmium tetroxide in sodium cacodylate buffer for 2 h at 25°C, washed with distilled water, and dehydrated in a series of ethanol. The specimens were coated with gold and were observed using a Jeol JSM-840A SEM.

**Antibody Staining**

Dechorionated embryos were stained using the protocol of Singh and Gopinathan, (1997). We used the Drosophila Dll antibody (Panganiban et al., 1995), which cross-reacts to Dll protein from other species (Beldade et al., 2002; Panganiban et al., 1995) and Drosophila Extradenticle (Exd) antibody (a gift from Rob White). Secondary antibodies (Jackson Laboratories) were Goat antirabbit IgG conjugated to FITC (1:200) and goat anti-mouse IgG conjugated to Cy-3. The large size, thickness, and auto-fluorescence of silkworm embryo prevented higher magnification images.

**Fate Mapping of Larval Legs by Surgical Manipulations**

Perturbations were performed on all three thoracic legs on the right side of the larvae in the first-, second-, third-, fourth- and fifth-instars, whereas the legs on the contralateral (left) side served as controls. Larvae were water anaesthetized (Kango-Singh et al., 2001) and a fine inci-

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sion was done on the outer thick cuticle of the thoracic legs using a surgical blade. The inner leg tissue beneath was exposed and excised. A small amount of phenylthiourea was applied to the epidermal opening after surgical manipulation to prevent oxidation of the haemolymph, which leads to the death of the larvae (Kango-Singh et al., 2001). Upon recovery from water anesthesia, the larvae were reared under standard conditions. Larvae earlier than second instar could not survive excisions and died due to excessive loss of haemolymph during recovery period. The following perturbation/excisions were performed on the larval legs:

1. In-situ Perturbation: The small portion of the leg tissue along the proximo-distal axis was exposed after incision in the larval leg and was teased/removed by a needle.

2. Segmental Excisions: The inner larval leg tissues were excised along the proximo-distal axes to remove various segments of larval leg using a surgical blade. The incisions on the legs were closed after the perturbations. These larvae were reared to adulthood and consequences of these leg excisions were studied in the adult moth. These manipulations were classified as follows:

a. Distal: The distal tip of larval leg corresponding to segments IV, and IV and V were excised (Fig. 4e).

b. Medial: The medial portion corresponding to segment III of the inner larval leg tissue was excised (Fig. 4f).

c. Proximal: The inner larval leg tissue corresponding to segment I and II were excised.

In another set of experiments, both the thicker outer leg cuticle and the inner leg tissue were excised at positions similar to a, b and c. Even though, such manipulations caused very high lethality and the phenotypes were similar.

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LITERATURE CITED


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