Functional analyses of *tiptop* and *Antennapedia* in the embryonic development of *Oncopeltus fasciatus* suggests an evolutionary pathway from ground state to insect legs

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Summary

In insects, selector genes are thought to modify the development of a default, or ‘ground state’, appendage into a tagma-specific appendage such as a mouthpart, antenna or leg. In the best described example, *Drosophila melanogaster*, the primary determination of leg identity is thought to result from regulatory interactions between the Hox genes and the antennal-specifying gene *homothorax*. Based on RNA-interference, a functional analysis of the selector gene *tiptop* and the Hox gene *Antennapedia* in *Oncopeltus fasciatus* embryogenesis is presented. It is shown that, in *O. fasciatus*, *tiptop* is required for the segmentation of distal leg segments and is required to specify the identity of the leg. The distal portions of legs with reduced *tiptop* develop like antennae. Thus, *tiptop* can act as a regulatory switch that chooses between antennal and leg identity. By contrast, *Antennapedia* does not act as a switch between leg and antennal identity. This observation suggests a significant difference in the mechanism of leg specification between *O. fasciatus* and *D. melanogaster*. These observations also suggest a significant plasticity in the mechanism of leg specification during insect evolution that is greater than would have been expected based on strictly morphological or molecular comparisons. Finally, it is proposed that a *tiptop*-like activity is a likely component of an ancestral leg specification mechanism. Incorporating a *tiptop*-like activity into a model of the leg-specification mechanism explains several mutant phenotypes, previously described in *D. melanogaster*, and suggests a mechanism for the evolution of legs from a ground state.

Key words: *Oncopeltus fasciatus*, *tiptop*, *Antennapedia*, Leg development, Ground state, RNA-interference

Introduction

The diversity of arthropod appendage types is thought to be controlled, in part, by selector genes. Selector genes encode transcription factors that, in combinations, define the body plan by controlling the development of specific regions of the embryo, and have been implicated in the evolution of body plan variations (Averof and Akam, 1995; Carroll et al., 2001; Duboule, 1994; Gellon and McGinnis, 1998; Manak and Scott, 1994; Panganiban et al., 1995; Rogers et al., 2002; Warren et al., 1994). In insects, selector genes are thought to modify the development of a default, or ‘ground state’, appendage into a tagma-specific appendage such as a mouthpart, antenna or leg.

The primary model organism for insect appendage formation is the dipteran *D. melanogaster*, in which regulators of leg development have been well characterized. In *D. melanogaster*, combinations of selector gene activity control the segmentation and differentiation of leg segments (Fig. 7) to produce coxa, trochanter, femur, tibia, tarsi, and pretarsus (proximal to distal). In brief, *extradenticle* (*exd*) and *teashirt* (*tsh*) are required for proximal development (Abu-Shaar and Mann, 1998; Erkner et al., 1999; Wu and Cohen, 2000); *Distalless* (*Dll*) is required for distal development (Cohen et al., 1989), and *dachshund* (*dac*) is required for medial development (Mardon et al., 1994). While these genes are required for elaboration of the axis of the leg and for normal leg development, the primary modifiers of ground state development are thought to be the Hox genes and *homothorax* (*hth*). These latter genes act as switches that choose among the various appendage types (Casares and Mann, 2001; Kaufman et al., 1990; Struhl, 1982, 1981).

The role of *Antennapedia* (*Antp*) in specifying leg development is typical of the Hox genes of *D. melanogaster*. *Antp* activity is required for segmentation of the medial region of the leg and for the specification of proximal, medial and distal leg identities (Struhl, 1982; Struhl, 1981). In the medial and distal region of the leg, the choice of identity is achieved by repressing the activity of the antennal specifier, *hth* (Casares and Mann, 2001; Yao et al., 1999). In the absence of *Antp* activity, *hth* activity expands distally and specifies the development of an antenna-like appendage, but with tarsal claws (pretarsi) at the tip (Casares and Mann, 2001; Dong et al., 2000; Struhl, 1981; Struhl, 1982). In addition to specifying antennal identity, *hth* is also required for segmentation of the
medial and proximal region (Casares and Mann, 2001). Removing the activities of both genes thought to control the choice of appendage identity (Antp and hth) causes leg-like appendages to form (Casares and Mann, 2001) that are divided into a distal region composed of normal tarsi and pretarsi and into a proximal region that is produced by a fusion of the more proximal segments (tibia, femur, coxa and trochanter).

Here we present the functional analyses of two selector genes (tiptop, Antp) that control leg development in the milkweed bug Oncopeltus fasciatus (Hemiptera). We show that, in O. fasciatus, tiptop is a selector gene that is required for the segmentation of the distal leg and is also required to switch appendage development from antenna to leg. We also show that, while the role of Antp in the segmentation of the medial leg is conserved between D. melanogaster and O. fasciatus, its role in specifying leg versus antennal development is not. The implication is that, in O. fasciatus, it is tiptop and not Antp which represses hth activity in the leg. We discuss the significance of the regulatory variation in the leg specification mechanism on the evolution of insects and arthropods.

Materials and methods

The gene tree of tsh-like genes was constructed using PAUP* (Swofford, 1996). Care for insect populations, methods of embryo collection, RNA extraction, whole-mount in situ hybridization of RNA probes, light and SEM microscopy are described in Rogers et al. (Rogers et al., 1997). The general method for RT-PCR cloning of tiptop, Antp and Ubx followed that of Peterson et al. (Peterson et al., 1999).

Cloning of tiptop used the forward primer GTNTGGYTGNNAAARGG (VWLKGY) and the reverse primer TTRTCRCANACYTTRCA (CKVCDC). Cloning of Antp used the forward primer GAYTAYCCNTTGGATGMGN (DYPWMR) and the reverse primer CKNCKRTTYTGRAACCA (WFQNRR). Cloning of Ubx used the forward primer GARYTNGARAARGARTTY (ELEKEF) and the reverse primer CKNCKRTTYTGRAACCA (WFQNRR).

RNA-interference (RNA-I) was performed generally as described in Hughes and Kaufman (Hughes and Kaufman, 2000). In brief, sense and antisense single-stranded (ss)RNAs were generated from cDNA clones by MEGASCRIPT (Ambion), extracted with phenol-chloroform, ethanol precipitated, and resuspended in injection buffer (0.1 mM NaPO4, 5 mM KCl, pH 6.8). The double-stranded (ds)RNA was produced by combining complementary ssRNAs in roughly equimolar ratios, heating at 95°C (1 min), and allowing the heat block to cool to room temperature. Production of dsRNA was verified by briefly treating samples of both ssRNA and dsRNA with weak RNAse A, and separating the products in 2% agarose gels briefly treating samples of both ssRNA and dsRNA with weak RNAse A, and separating the products in 2% agarose gels

Results

To investigate the variation and evolution of mechanisms of insect appendage formation, we examined the role of selector genes in the formation of embryonic appendages of O. fasciatus. There are several advantages in using this species to study leg development and evolution. First, as a hemimetabolous insect, O. fasciatus first instars have fully formed legs (i.e. all segments are present). Thus, the entire process of leg formation is readily apparent in the embryo and is not stretched out over the process of imaginal disc formation and metamorphosis as it is in holometabolous insects such as D. melanogaster. Second, O. fasciatus is positioned more basally on the insect phylogeny (Kristensen, 1991) than any other insect for which the RNA-I technique has been successful in assaying gene function. Third, because O. fasciatus is more distantly related to D. melanogaster than the more common genetically tractable model insects (e.g. the holometabolous insects Tribolium castaneum and Bombyx mori), there is greater potential for uncovering regulatory variation and perhaps gaining greater insight into the evolution of the leg specification mechanism. Fourth, because O. fasciatus is generally less derived and has an ancestral leg composition, it may also have conserved the ancestral mechanisms of leg development allowing the direction of evolutionary change to be inferred.

From O. fasciatus embryos, we cloned partial cDNAs that represent a homolog of the D. melanogaster gene tiptop (Fig. 1). tiptop is a member of the tsh-family that is typified by zinc-finger motifs and is presumed to be a transcription factor (Andrew et al., 1994; Röder et al., 1992) (GenBank Accession Number, AF219383). Also, while present in the D. melanogaster genome, this gene was identified solely on the basis of molecular data. No mutations have been reported in tiptop, and its developmental function has not been previously reported for any arthropod.

Accumulation of tiptop mRNA during embryogenesis

The tiptop cDNAs were used as a probe for in situ hybridization to determine the pattern of mRNA accumulation during embryogenesis (Fig. 2). The initial accumulation of tiptop mRNA is strongest in the thorax (Fig. 2A). During germ band elongation, it covers the entire thorax (dorsal to ventral,
proximal to distal) and includes the leg primordia (Fig. 2B). Later in fully elongated embryos, the accumulation in the legs modulates (Fig. 2C,E), forming anteriorly positioned arcs of higher and lower accumulation and becoming absent from the distal tip. 
tiptop mRNA also accumulates in the gnathos (Fig. 2B), the procephalon (Fig. 2D) and in the caudal region (Fig. 2C). This expression pattern is similar to that of 
tiptop in Thermobia domestica (Peterson et al., 1999), an apterygote that lies most basally on the insect phylogenetic tree (Kristensen, 1991) in the sister taxa of pterygotes (Thysanura-Zygentoma).

The similarity of the two expression patterns and their position on the phylogenetic tree support the hypothesis that the similarities in 
tiptop expression between the two insects represent elements of the ancestral 
tiptop expression pattern.

**Injection of 
tiptop dsRNA produces specific defects**

To gain insight into the function of several O. fasciatus genes on the development of the larval cuticle, gene activity was reduced using RNA-I. RNA-I was performed by injecting in vitro synthesized dsRNA corresponding to a specific cDNA into precellular embryos. The developmental effects of injecting specific dsRNAs were compared to control groups consisting of: (1) eggs collected and prepared but not injected, and (2) group of eggs which are injected with buffer or mock transcription reactions. Developmental defects detected in group 1 would reveal any natural variation in development or defects in development caused by manipulation of the eggs. Nearly all of these eggs hatch with normal phenotypes (data not shown), except for some unfertilized eggs which are easily recognized and are not included in the other experimental regimes. The developmental defects observed in group 2 reveal the effects of damaging the eggs by injection and the effects of transcription reagents that may remain in the injection mix. Common defects in this group include randomly positioned deletions which we attribute to partial losses of the embryo from the egg during injection. Specific defects caused by the injection of a particular dsRNA are defects which are observed in repeated injections and are not observed in the control populations. These specific defects are presumed to be the phenocopies caused by reductions in gene activity. The collective defects caused by injection of dsRNA from a particular gene are usually different from those caused by dsRNA from other genes, suggesting a reduction in activity of a specific gene.

The results of RNA-I analysis for 
tiptop and several other genes are summarized in Table 1. The table shows that the percentage of developing animals showing a specific defect is reasonably high (approximately 25-40%) and that the specific defect associated with reducing the activity of each gene is unique.

In our RNA-I analysis, we observed variable strengths of the presumed 
tiptop phenocopy, which we arbitrarily divided into two grades: moderate (viable) and strong (lethal). We interpret this range of morphology as resulting from a range of gene inactivation from partial to near complete loss of function, although in our analysis, we cannot rule out residual gene activity present in even the most strongly affected animals. While divided into two grades, the affected animals

![Fig. 2. The embryonic accumulation of 
tiptop mRNA is revealed by in situ hybridization. (A) mRNA accumulation is first detected in the thoracic (T), labial (Lb) and maxillary (Mx) segments of the elongating germband and is highest in the thorax. During germband extension (B), mRNA can be detected in the procephalon (D) including the labral (Lr), antennal (An) and ocular (Oc) segments. (C) At the fully extended germband stage, mRNA accumulates in the caudal (cad) region and weakly in the first abdominal (A1) and mandibular (Mn) segments. The darkening of the posterior abdomen in this animal results from nonspecific chromogen and unremoved yolk; it does not represent 
tiptop expression. (E) After germband extension, mRNA accumulation becomes mosaic in the legs. Anterior arcs of elevated accumulation (small arrows) form and regions of the distal tips lose expression completely (large arrow). Ic: intercalary segment.](image1)

![Fig. 3. 
tiptop RNA-I causes a range of embryonic defects that only affect the leg. (A) Control injections produce normal morphology. (B) Moderate 
tiptop phenocopies show segmentation abnormalities in the distal leg. (C) Strong 
tiptop phenocopies show distal legs transformed to antennae. Distal legs are marked with a white arrow. All other regions of the animals appeared normal based on general morphology and the presence of cuticular structures such as bristles, sense organs, and spiracles.](image2)
represent a continuum of the phenocopy and are interpreted in the same manner as an allelic series of mutations in traditional genetic analysis. Fig. 3 shows representative animals resulting from injections of *tiptop* dsRNA. Despite the broad expression pattern of *tiptop* mRNA, suppression of *tiptop* activity by RNA-I affects only the legs (Table 1). All other regions of the animals appeared normal based on general morphology and the presence of cuticular structures such as bristles, sense organs, and spiracles.

**tiptop is required for the segmentation of distal leg segments and acts as a switch between antennal and leg development**

Animals displaying the moderate *tiptop* phenocopy were characterized by tarsal segments that were reduced in size and by pretarsi (claws, augmentum, pulvilli) that were absent or reduced (compare Fig. 4A,B to Fig. 4C,D). Animals displaying the strong *tiptop* phenocopy were characterized by a complete absence of tarsi and pretarsi and the tibia was transformed toward distal antenna (compare Fig. 5A to Fig. 5C,D and Fig 6C). The antennal identity of the transformed tibia was verified by their general morphology, the presence of a bristle density gradient (not present in leg segments), and the presence of sensory pegs normally found only on antennae (compare Fig. 5B to Fig. 5E,F). The strong *tiptop* phenocopy also causes a shortening of the femur (Fig. 6C).

We interpret these phenocopies to mean that, in *O. fasciatus*, *tiptop* is required for the production of the pretarsi and for the segmentation of the distal leg (tibia-pretarsal). Legs with reduced *tiptop* activity fail to form the segment borders between tibia and tarsi and between the tarsi. Whether the cells in the unsegmented region are lost, by either failure of cell division or cell death, or if these cells simply become fused into a composite segment is unclear. However, the size of the remaining segments in larvae and examinations of the embryonic legs of animals that are likely to display a *tiptop* phenocopy must be considered as a result of reduced *tiptop* activity.

### Table 1. Summary of RNA-I experiments

<table>
<thead>
<tr>
<th>Gene*</th>
<th>Number of embryos injected</th>
<th>Number with a specific defect†</th>
<th>Injected animals completing development (%)</th>
<th>Animals with a specific defect (%)</th>
<th>Specific defect</th>
<th>Wild-type function</th>
</tr>
</thead>
<tbody>
<tr>
<td>tiptop</td>
<td>969</td>
<td>52</td>
<td>19.4</td>
<td>27.6</td>
<td>Fusion of distal leg, distal leg to antenna</td>
<td>Segmentation and specification of distal leg</td>
</tr>
<tr>
<td>Antp</td>
<td>418</td>
<td>64</td>
<td>39.7</td>
<td>38.5</td>
<td>Fusion of medial and distal leg segments</td>
<td>Segmentation and specification of medial and distal leg</td>
</tr>
<tr>
<td>Dfd</td>
<td>551</td>
<td>65</td>
<td>30.1</td>
<td>39.1</td>
<td>Deformed maxilla, mandible to antenna</td>
<td>Specification of maxilla and mandible</td>
</tr>
<tr>
<td>Scr</td>
<td>481</td>
<td>39</td>
<td>32.6</td>
<td>24.8</td>
<td>Labium to antenna, loss of T1 leg comb</td>
<td>Specification of labium and leg comb</td>
</tr>
<tr>
<td>Ubx</td>
<td>300</td>
<td>23</td>
<td>29.7</td>
<td>25.8</td>
<td>Ectopic appendage and sternite in A1, A1 and T3 to T2</td>
<td>Specification of A1 and T3</td>
</tr>
<tr>
<td>Antp+tiptop</td>
<td>718</td>
<td>49</td>
<td>26.7</td>
<td>25.5</td>
<td>Fusion of medial and distal leg segments, leg to antenna</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>975</td>
<td>0†</td>
<td>42.5</td>
<td>0‡</td>
<td></td>
<td>Controls are not considered specific defects.</td>
</tr>
</tbody>
</table>

*Gene that corresponds to the dsRNA injected
†Number of embryos that produced a repeatable defect not seen in control injections of buffer
‡By definition, defects seen in controls, usually deletions, are not considered to be specific defects.
Control refers to embryos injected with buffer and mock transcription reactions.
T1, T2, T3: first, second and third thoracic segments.
A1: first abdominal segment.

*Fig. 4.* The moderate *tiptop* phenocopy shows a loss of pretarsi and reduction of tarsi (C,D). (A) A leg of a buffer-injected animal displaying a normal phenotype. (B) Detail of the pretarsus in (A), the arrow marks the tarsus-tibia junction in A,C,D. Fe: femur, Ti: tibia, Ta: tarsus.
Development genes can establish leg versus antennal identity in leg identity specification functions because the activity of Hox melanogaster tip top specifying leg development in reduced Hox function. We examined the role of Hox genes in several significant differences in their activity compared to RNA-I. Our analysis of Hox genes in and trochanter as well as by a third segment (DM*; produced Antp the strong phenotype suggests that loss and segment fusion are occurring. There are femur—by the fusion of the medial and distal segments) that is tipped with normal, tip top-dependent pretarsi (Fig. 6B, Table 1). All three pairs of legs are affected equally.

In O. fasciatus, reducing the activities of both Antp and tip top results in legs composed of normal proximal segments and a third segment (DM*; femur+tibia+pretarsi) that has an antennal identity (Fig. 6D, Table 1). Reducing the activity for two other Hox genes that control leg development in D. melanogaster, Sex combs reduced (Scr) and Ultrabithorax (Ubx), produces little or no effect on leg morphology (Table 1).

We interpret our results to mean that Scr and Ubx are not specifiers of leg development in O. fasciatus. Also, Antp activity is required for the development of distal and medial segments, but not for the proximal leg or pretarsi. Further, in contrast to D. melanogaster, where loss of Hox (Antp, Scr, Ubx) function produces dramatic transformations of leg to antenna (Struhl, 1982; Struhl, 1981), we detected no transformation toward antennae of Antp-phenocopies legs. Finally, in O. fasciatus, reducing the activities of both Antp and tip top simultaneously had a strictly additive effect. Thus, there is no evidence for a genetic interaction between tip top and Antp.

Discussion

Tip top is a selector gene involved in leg segmentation and specification of distal identity

Our results demonstrate that, in O. fasciatus, tip top is required for distal (tibia-pretarsal) leg segmentation and differentiation. The transformation of the most distal remaining leg segment toward distal antenna in tip top phenocopies suggests that tip top is a selector gene that acts as a specifier of leg identity. Among many possible explanations of this transformation is that, like D. melanogaster, repression of an antennal specifier is required for the development of leg identity. We suggest that, in the presence of lowered tip top activity, the activity of an antennal specifier expanded into the distal portion of the affected legs where it directed antennal development. If we assume that hth acts as the antennal specifier in O. fasciatus as it does in D. melanogaster; then tip top may function, in part, by repressing the activity of hth (Fig. 7).

Expression of tip top mRNA over the distal thoracic appendage during early stages of embryonic development is consistent with its activity in the leg. Modulation of the leg expression pattern in later stages seems consistent with tip top’s role in segmentation. However, the loss of expression in the distal region of the leg suggests that the effect of tip top on distal identity results from a transient and indirect activity. It may be that all the defects in the tip top phenocopies result from misregulation of a single gene such as hth. Alternatively, the segmentation and distal specification function of tip top may result from a more direct role in stabilizing or modulating distally required genes such as Dll or EGFR (Campbell, 2002; Galindo et al., 2002).

The restriction of tip top activity to the leg is somewhat surprising given its broad mRNA expression pattern and the conservation of this pattern to T. domestica. This conserved mRNA expression pattern may simply be unnecessarily broad, or some tip top activity may be redundant, or the pattern may reflect a requirement for undetected (e.g. not cuticular) activities of tip top. Such activities are suggested by the lethal

Fig. 5. The strong tip top phenocopy displays a transformation of distal leg to antennae. (A) A normal antenna. (B) Close up of (A) showing antennal-specific sensory pegs (arrow). (C-F) Legs displaying the strong phenocopy. Tarsi and pretarsi are absent. The presumptive tibia (Ti) is the most distal segment and is transformed toward antenna based on its shape, a bristle density gradient and the presence of the antennal-specific sensory pegs (arrows in E,F). Fe: femur.

Antp does not act as a switch between antennal and leg development in O. fasciatus

We considered the Hox genes to be candidates for genes with leg identity specification functions because the activity of Hox genes can establish leg versus antennal identity in D. melanogaster, and because the leg morphology of the strong tip top phenocopy was similar to that of D. melanogaster with reduced Hox function. We examined the role of Hox genes in specifying leg development in O. fasciatus embryos using RNA-I. Our analysis of Hox genes in O. fasciatus suggests several significant differences in their activity compared to their activity in D. melanogaster.

In O. fasciatus, the moderate Antp phenocopy is typified by a distortion or a fusion of the medial segments (M*; produced by the fusion of femur and tibia, in Fig. 6A). Here the fusion, rather than loss of segments is more apparent. There are femur-like sense organs on the proximal edge of the segment and (on first thoracic legs) a tibial comb on the distal edge. The legs of the strong Antp phenocopy are characterized by a normal coxa and trochanter as well as by a third segment (DM*; produced by the fusion of the medial and distal segments) that is tipped with normal, tip top-dependent pretarsi (Fig. 6B, Table 1). All three pairs of legs are affected equally.

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**Fig. 6.** Antp and tiptop are required for distal and medial development. (A) The weak Antp phenocopy has an apparent fusion of medial segments (M³=Fe+Ti). (B) The strong Antp phenocopy has a fusion of medial and distal (DM³=Fe+Ti+Ta). (C) The strong tiptop phenocopy has a fusion of distal segments (D³=Ti+Ta) and D³ has an antennal identity. All appendages have normal coxa (Cx) and trochanter (Tr) that can be identified by unique sense organ clusters (arrows in A,B,D) found at the proximal edges of the segments. Pt: pretarsus.

**A comparison of the tsh-like genes of insects suggests a recent duplication in the lineage leading to Drosophila**

As illustrated in Fig. 1, tsh-family genes have been cloned by different methods from at least five insect species and, with the exception of *D. melanogaster* tsh, all appear to have greater similarity to tiptop. Through extensive PCR on genomic DNA and cDNA, we recovered both tiptop and tsh from *D. melanogaster*, but we only recovered only a single gene from *O. fasciatus* or other insects. Also, a gene tree (not shown) constructed using PAUP* (Swofford, 1996) for the tsh-family genes shows that the two *D. melanogaster* genes cluster together. The gene tree and the absence of a tsh gene in the other insects surveyed suggest that the two tsh-family genes in *D. melanogaster* result from a recent duplication of an ancestral gene. We call this ancestral gene tiptop because of its greater similarity to that gene and not to imply a closer evolutionary relationship of the ancestral gene to either the *D. melanogaster* tsh or tiptop.

*tsh* has a variety of developmental functions in the cuticle of the *Drosophila* larva and adult. Specifically, *tsh* is thought to specify trunk identity in the larva through interactions with Hox genes (Röder et al., 1992), is required for the formation of proximal regions of appendages in adults (Abu-Shaar and Mann, 1998; Erkner et al., 1999; Wu and Cohen, 2000), and plays a role in restricting the development of the adult eye (Bessa et al., 2002; Singh et al., 2002). There is little similarity between any of these activities of the *Drosophila* tsh gene and *O. fasciatus* tiptop. Thus, these activities appear to have been acquired by the tsh-family relatively recently. Significant to the discussion here is that the function *tsh* has in the formation of the proximal region of the leg in *D. melanogaster* cannot be provided by *tsh* in *O. fasciatus* because the gene is not present. These roles may be provided by other proximally expressed genes such as *hth* and *exd*.

**Variation in the genetic mechanism of leg specification among insects**

A model of the leg specification mechanisms in *D. melanogaster* and the proposed differences from *O. fasciatus* are shown in Fig. 7. In contrast to *D. melanogaster*, where loss of Hox (*Antp, Sc*, *Ubx*) function produces dramatic transformations of leg to antenna (Struhl, 1981; Struhl, 1982), we detected no transformation toward antennae of *Antp-
phenocopy legs that could be interpreted as an expansion of hhh activity. Thus, although it is possible that some residual Antp function remains in our animals, we suggest that it is tiptop and not Antp that represses the activity of hhh (or other antennal specifier) in the O. fasciatus leg. A role for Antp in the segmentation of the distal region is absent in D. melanogaster while its role in medial segmentation is conserved between the two insects. Also, given that neither tiptop nor the Hox genes act as specifiers of proximal identity (leg vs. antenna) in the leg specification mechanism of milkweed bugs, additional undetermined genes are implicated. This further distances the mechanism of appendage specification in milkweed bugs from the relatively simple two-gene (Hox, hhh) system evident in D. melanogaster.

A tiptop-like activity is also evident in D. melanogaster. This is illustrated most convincingly by the persistent pretarsi formed on legs that are otherwise transformed to antennae in the absence of Antp activity (Struhl, 1981; Struhl, 1982). Also, the leg-like appendage (composed primarily of tarsi and pretarsi) produced by hhh Antp null clones in D. melanogaster (Casares and Mann, 2001) is what might be predicted if an independent tiptop-like activity for distal segmentation and specification remained active in these appendages. Genetic analysis of Drosophila tiptop has not revealed a role in distal specification or segmentation of the adult leg (Laurent Fasano, personal communication). However, due to the technical difficulties of determining the role embryonic gene activities have in adult structures in D. melanogaster, it has not been possible to rule out that embryonic activities of either tiptop or tsh affect the adult leg. Thus, it remains a possibility that a tiptop-like activity could be provided by tiptop or tsh, as well as by other genes in D. melanogaster.

Interestingly, the defects induced by reduced Antp activity in O. fasciatus are in striking contrast to the transformations of mouthparts to antennae seen when Scr and Dfd activity are reduced (Table 1) (Hughes and Kaufman, 2000). These latter transformations have been used as evidence for a universal mechanism of Hox specification of insect appendages (Hughes and Kaufman, 2000; Casares and Mann, 2001). However, in O. fasciatus, Scr and Dfd apparently repress the activity of antennal specifier gnathal appendages while Antp does not repress this activity in thoracic appendages. Thus, in O. fasciatus, two mechanisms (Hox-dependent and a Hox-independent) exist for specifying the identity of appendages. Additional factors (including tiptop) must mediate the differences in the active mechanisms in these tagns.

It should be noted that, while reduced Scr and Ubx activity cause segmental transformations of identity (labial and A1, respectively), the absence of such a transformation by reduced Antp is not unexpected. The primary and most frequent transformation caused by loss-of-function (hypomorphic and null) Antp alleles of D. melanogaster is of the second thoracic segment (T2) toward the first thoracic segment (T1). This is frequently seen as the formation of a T1-specific structure, the denticle beard, in T2 (Wakimoto and Kaufman, 1981). In our analysis, T1 and T2 of the first instar milkweed bug are homomorphic (except for the Scr-dependent leg comb) (Hughes and Kaufman, 2000). Therefore, a T1 to T2 transformation in milkweed bugs, as might be expected for reduced Antp function, would be undetected at the level of the cuticle. Other cuticle defects produced by loss-of-function Antp alleles in D. melanogaster include disruptions in the denticle band pattern, the formation of sclerotized tissue, and occasional rudimentary denticle beards in T3. We did not observe comparable defects in our investigations with O. fasciatus. However, their apparent absence may be due to a low frequency of these defects, insufficient knowledge of the detail of the O. fasciatus cuticle, or residual Antp activity remaining in our experimental animals.

**Evolution of the genetic mechanism for leg specification**

Our discovery of significant variation in the mechanism of leg specification can be added to a growing number of examples that suggest there is greater regulatory diversity in the mechanisms underlying development than could have been suspected from the high conservation of the regulatory genes themselves or the processes they regulate. In this case, both O. fasciatus and D. melanogaster form legs of similar composition, and thus the processes of segmentation and specification are conserved. However, the regulatory mechanisms governing these processes are not conserved. This regulatory variation suggests how developmental mechanisms can evolve (using regulatory changes, gene duplication, and divergence) without compromising the organism. It is this kind of genetic variation that can provide significant raw material for morphological evolution.

A path of evolutionary change, and possible transitional states, in the leg specification mechanism can be inferred by assuming that the ancestral state of insect leg development is better represented by O. fasciatus than by D. melanogaster. This assumption is reasonable, given that O. fasciatus appears to have retained the ancestral states of embryonic leg formation and two tiptop-related characters (i.e. genome content of tsh-family members and expression pattern). Combined with its more basal position on the phylogenetic tree, O. fasciatus seems more likely than D. melanogaster to have conserved the ancestral mechanisms of leg formation.

Therefore, we can describe the genetic changes required for the O. fasciatus mechanism of leg development to evolve into that of D. melanogaster. First, Antp acquired the ability to repress the antennal specifier (hhh) in the distal leg and lost its role in distal segmentation. These changes might have been relatively simple. A mechanism for Hox genes (e.g. Scr and Dfd) to repress the antennal specifier already existed and the segmentation functions of Antp might be partially provided by tiptop. Second, the change in Antp function relaxed the constraints on tiptop, thereby allowing its function (including hhh repression) to diverge. Finally, duplication and further divergence of the ancestral tiptop gene produced the tsh and tiptop genes of D. melanogaster.

**Genetic evolution of leg specification from a ground state**

The variation we have detected in the genetic control of leg development, and specifically that the segmentation and specification activities of independent genes evolve independently, impacts genetic models of the ground state appendage and the evolution of its variations. By definition, the ground state appendage is formed in the absence of all appendage-modifying selector genes. In our proposed model
(Fig. 7), the activities of at least three genes (Antp, hth, and a tipiop-like activity) in D. melanogaster; and perhaps additional genes in O. fasciatus, need to be eliminated to recreate the ground state.

At this time, such a recreation has not been accomplished in any insect and the morphology of a recreated ground state appendage is difficult to predict. Significantly, even when formed in the absence of identity specifier activity, an appendage of an extant insect might have the identity of a ground state appendage, but it is unlikely to have the morphology of an ancestral ground state appendage because the segmentation and identity-specifying activities of selector genes evolve independently.

Although a recreated ground state that is leg-like or antenna-like would suggest that insect antennae are evolutionary modifications of primitive leg-like structures (or vice versa), one that has neither identity would suggest that both antennae and legs could be independent modifications of a more basic appendage type. Based on the requirement for segmentation activities in all appendage types, as well as the presence of distinct cephalic and thoracic appendages among all arthropods, we suggest a model in which the cephalic and thoracic appendages diverged from a segmented, but undifferentiated ground state. This divergence appears to predate the evolution of insects and it may be shared by all modern arthropods. In our example, the segmentation activities of the appendage selector genes evolved first, and the identity functions of the selector genes evolved later. Thus, in this example, the variable types of appendages would have evolved only after multisegmented, but relatively undifferentiated appendages were present.

Only further genetic investigations of additional genes in a greater variety of arthropod species will clarify the genetic evolution of appendage diversification. These investigations could verify the existence of a common appendage ground state and help identify the ancestral specifiers of appendage segmentation and identity in arthropods.

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