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Leg development in flies versus grasshoppers: differences in *dpp* expression do not lead to differences in the expression of downstream components of the leg patterning pathway

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SUMMARY

All insect legs are structurally similar, characterized by five primary segments. However, this final form is achieved in different ways. Primitively, the legs developed as direct outgrowths of the body wall, a condition retained in most insect species. In some groups, including the lineage containing the genus *Drosophila*, legs develop indirectly from imaginal discs. Our understanding of the molecular mechanisms regulating leg development is based largely on analysis of this derived mode of leg development in the species *D. melanogaster*. The current model for *Drosophila* leg development is divided into two phases, embryonic allocation and imaginal disc patterning, which are distinguished by interactions among the genes *wingless (wg)*, *decapentaplegic* (*dpp*) and *distalless (dll)*. In the allocation phase, *dll* is activated by *wg* but repressed by *dpp*. During imaginal disc patterning, *dpp* and *wg* cooperatively activate *dll* and also indirectly inhibit the nuclear localization of Extradenticle (Exd), which divide the leg into distal and proximal domains. In the grasshopper *Schistocerca americana*, the early expression pattern of *dpp* differs radically from the *Drosophila* pattern, suggesting that the genetic interactions that allocate the leg differ between the two species. Despite early differences in *dpp* expression, *wg*, Dll and Exd are expressed in similar patterns throughout the development of grasshopper and fly legs, suggesting that some aspects of proximodistal (P/D) patterning are evolutionarily conserved. We also detect differences in later *dpp* expression, which suggests that *dpp* likely plays a role in limb segmentation in *Schistocerca*, but not in *Drosophila*. The divergence in *dpp* expression is surprising given that all other comparative data on gene expression during insect leg development indicate that the molecular pathways regulating this process are conserved. However, it is consistent with the early divergence in developmental mode between fly and grasshopper limbs.

Key words: *Schistocerca americana*, Leg development, *decapentaplegic*, Imaginal disc evolution

INTRODUCTION

Morphologically, *Drosophila* is characterized by a highly derived mode of leg development. Both the development of adult legs from imaginal discs and the allocation of discs during embryogenesis, rather than during the last larval instar, are derived within the Diptera (Truman and Riddiford, 1999). Primitively in insects, all appendages developed as direct outgrowths of the body wall. This mode of development is retained in all ametabolous and hemimetabolous species and in many holometabolous species. Although the adult appendages resulting from direct development and from imaginal discs are similar, the appendages differ developmentally in important ways, including tissue architecture and relative timing of development, both of which may have consequences for molecular patterning.

A sophisticated model for the development of *Drosophila* legs has emerged during the last few years; however, the applicability of this model to other species, including those with direct development of legs, remains largely unexplored. *Drosophila* leg development can be separated into two phases, each controlled by distinct patterns of gene regulation. Early in embryonic development, leg discs are allocated from the embryonic ectoderm via an interaction between the genes that pattern the anteroposterior (A/P) and dorsoventral (D/V) axes of the embryo. Leg imaginal discs are positioned at the boundary between *wg* and *engrailed* (*en*)/*hedgehog* (*hh*) expressing cells along the A/P axis (Diaz-Benjumea et al., 1994). Along the D/V axis, DER (the *Drosophila* epidermal growth factor receptor homologue) is essential for restricting the leg primordia ventrally (Raz and Shilo, 1993) and the *dpp* gene acts to inhibit distal leg development dorsally (Goto and Hayashi, 1997). Wg activates

dll in a circular cluster of cells at the boundary between *wg* and *en/hh* expression and at the ventral edge of *dpp* expression (Cohen, 1990; Diaz-Benjumea et al., 1994). *dll* is essential for outgrowth of legs in *Drosophila* as null mutants show loss of the distal leg structures up to the proximal leg segment (Cohen et al., 1989). At the end of this allocation phase of leg development, imaginal discs are segregated from the remainder of the embryo as small invaginations that lie beneath the ectoderm.

A second phase of leg development, which we refer to as imaginal disc patterning, occurs during larval and pupal development. Elaboration of the P/D axis, which involves some of the same genes used to allocate leg primordia, occurs during this phase. Wg and Dpp are thought to act cooperatively as morphogens to activate or repress target genes in discrete domains along the P/D axis (Lecuit and Cohen, 1997; Wu and Cohen, 1999). In the leg imaginal discs, *wg* is expressed at high levels ventrally and *dpp* at high levels dorsally (Baker, 1988; Couso et al., 1993; Masucci et al., 1990). Thus, cells near the center of the disc (corresponding to distal limb segments) receive high levels of both Wg and Dpp while more peripheral cells (corresponding to proximal limb segments) do not. High levels of both Wg and Dpp activate Dll (Lecuit and Cohen, 1997) and repress Homothorax (Hth), another transcription factor (Wu and Cohen, 1999). Hth is expressed at high levels proximally where it binds to Extradenticle (Exd) and facilitates its movement into the nucleus. Thus, restriction of Exd function to the proximal region is an indirect result of Wg and Dpp signaling (reviewed by Morata and Sanchez-Herrero, 1999). Nuclearly localized Exd controls proximal leg patterning. Recently, Goto and Hayashi (1999) have argued that an additional intercalary mechanism is involved in patterning the intermediate regions of the leg.

How leg development is regulated in insects with other modes of development is not known. While it might be expected that the evolution of imaginal discs would be accompanied by substantial alterations in limb allocation and patterning, it could also be the case that the same mechanism is deployed in species with and without imaginal discs. The latter possibility would require a change in the timing of the elaboration of the P/D axis. Currently, what little is known about the molecular basis of appendage patterning in other insects suggests that leg patterning is remarkably conserved, even in insects with different modes of development (Niwa et

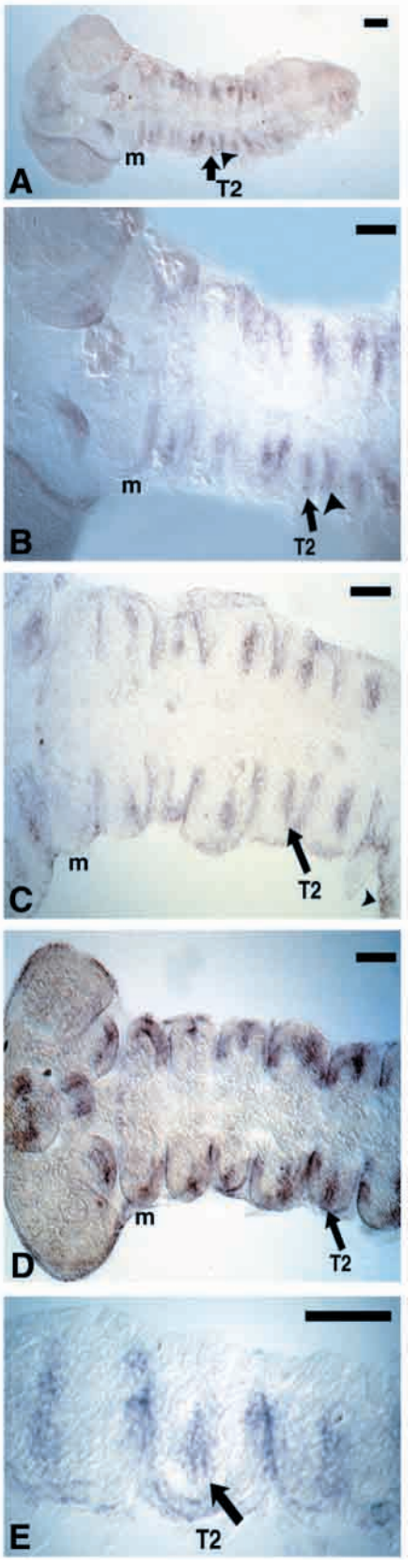

Fig. 1. *dpp* mRNA expression in early *S. americana* embryos (approx. 15-25% development). All embryos are viewed ventrally with anterior to the left. (A) <20% development. Grasshopper embryos develop gradually along both A/P and D/V axes. At this stage of development the head is visible as two large lobes at the anterior, followed by the gnathal and thoracic segments. Additional segments will be added sequentially, from the posterior end, as the embryo grows. The dorsal-ventral extent of the embryo is also quite limited at this stage, with the edges of the embryo at the top and bottom of the picture representing the most dorsal regions of the embryo. *dpp* stripes are present both intrasegmentally (arrow) and intersegmentally (arrowhead). (B) Higher magnification of embryo shown in A. (C) 20% development. *dpp* is expressed in a band along the dorsal (lateral) edge of the embryo (arrowhead); in the legs, staining has resolved to a pair of dominant intrasegmental stripes in each segment (arrow). (D) 25% development. The legs have begun to develop by direct outpocketing from the ventral surface (out of the plane of the image). *dpp* expression is detected intrasegmentally (arrow). (E) Higher magnification of the second thoracic segment shown in C. Scale bars, 20 μm. m, mandible, T2, second thoracic segment.

al., 1997; Panganiban et al., 1994; Nagy and Carroll, 1994; Sanchez-Salazar et al., 1996).

We have examined the expression of homologues of four *Drosophila* leg patterning genes – *wg*, *dpp*, Dll and Exd – in the hemimetabolous insect *Schistocerca americana*. The expression of *wg* mRNA appears as predicted based on *wg* expression patterns in *Tribolium* and *Drosophila*. (Baker, 1987; Nagy and Carroll, 1994). However, we detect major differences in the pattern of *dpp* expression. Nonetheless, the patterns of Dll and Exd, respectively a direct and an indirect target of Wg-Dpp regulation in *Drosophila*, closely parallel their expression patterns in *Drosophila*. This suggests a model for evolutionary changes in limb development wherein downstream events are conserved, while the upstream regulators are open to change. This model provides a mechanism for changes that occur early in an organism's life history, such as the evolution of imaginal discs.

MATERIALS AND METHODS

Grasshopper rearing and fixation

Schistocerca americana rearing and egg collection are described by Hunter (1961). Embryos were dissected in phosphate-buffered saline (1× PBS, pH 7.2), fixed in 4% formaldehyde in PEM (for antibody staining) or in 0.08 M Hepes, pH 6.9; 1.6 mM $MgSO_4$, 0.8 mM EGTA; 1× PBS, pH 7.2 (for in situ hybridization) for 45-75 minutes, and stored in 100% methanol at –20°C until use. Embryos were staged to the nearest 5% development according to Bentley et al. (1979).

In situ hybridization – *wg* and *dpp*

Digoxigenin-labeled sense and antisense riboprobes were synthesized from two sources: an *S. americana dpp* cDNA approximately 1.6 kb in length that contains 673 bp of coding sequence and roughly 1 kb of 3′ untranslated sequence (Newfeld and Gelbart, 1995) and a 538 bp *S. americana wg* PCR fragment provided by M. Friedrich (GenBank accession no. AF149776). The probes were hydrolyzed to an average size of 150 bp. Embryos were rehydrated, then heated at 75-80°C in hybridization buffer (50% formamide, 5× SSC, 100 μg/ml sheared salmon sperm DNA, 50 μg/ml heparin, and 0.1% Tween-20) for 30-60 minutes to inactivate endogenous alkaline phosphatases and at 60°C for 4 hours. Embryos were hybridized overnight at 60°C in 1 ng probe/μl hybridization buffer, washed over several hours at 60°C to remove excess probe, and blocked in 2% bovine serum albumin in PBS + 0.1% Triton X-100. Alkaline phosphatase-conjugated anti-digoxigenin antibody incubation and detection followed standard protocols (Nulsen and Nagy, 1999). Embryos were counterstained with 1 μg/ml DAPI (Sigma) to visualize nuclei, and mounted in 80% glycerol. Photographs were taken on a Zeiss Axiophot microscope with Ektachrome 160T film.

Antibody staining – Dll and Exd

The Dll and Exd antibodies were generous gifts from G. Panganiban and R. A. H. White respectively. The Exd antibody was preabsorbed overnight on fixed grasshopper embryos. Both antibodies were used at a 1:20 dilution; the antibody detection protocol was exactly as described by Panganiban et al. (1995) using a secondary antibody conjugated to Cy3 or Cy2 (Jackson Labs). Antibody stained embryos were counterstained with DAPI and analyzed and imaged on a Biorad 600 confocal microscope.

Drosophila GFP expression

dpp^{blk}/dpp^{blk}; dpp-blink.Gal4[39B2]/TM6,*Tb* flies were mated to flies homozygous for the UAS>superGFP[T2-1] transgene (kind gifts from L. Marsh). The UAS.superGFP[T2-1] transgene is described by Ito et al. (1997) and the dpp-blink.Gal4[39B2] driver is described by Staehling-Hampton et al. (1994). A subset of the larvae and pupae resulting from this cross express UAS.GFP driven by dpp-blink.Gal4. These larvae and pupae were identified by their non-Tb phenotype. GFP expression was examined in live, dissected leg discs on a Zeiss Axiophot microscope and a Biorad 600 confocal microscope.

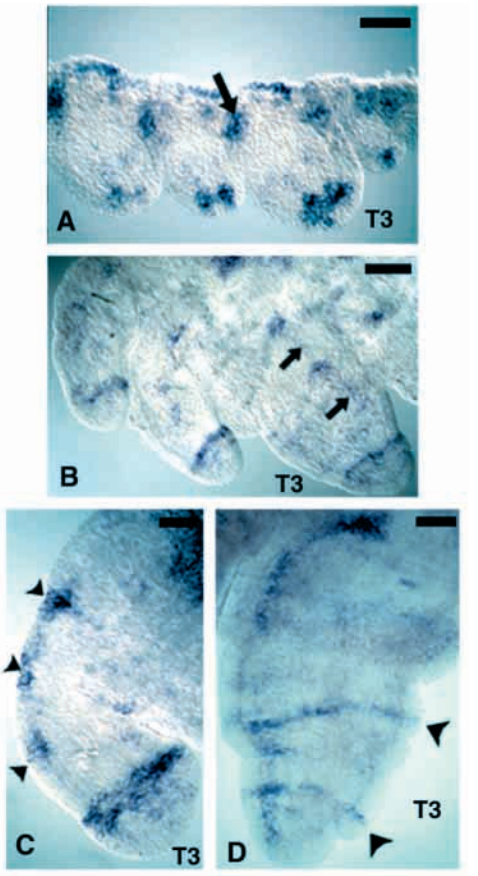

Fig. 2. *dpp* mRNA expression in the legs of mid-stage grasshopper embryos (<30-40% development). A and B show legs in lateral view with anterior to the left. C and D show dissected legs with dorsal to the left. (A) <30% development; *dpp* mRNA is detected along the dorsal band, in a pair of spots near the distal tip of each leg, and in an "armpit" patch (arrow). No staining is detectable along the dorsal edge of the leg. (B) 35% development; *dpp* is strongly expressed in a nearly circumferential ring close to the distal end of the appendage. In addition, there is a faint stripe along the dorsal length of the developing leg (arrows). (C) Similar stage to B. Nodes of higher expression in the dorsal stripe (arrowheads) may correspond to places where additional rings will form, as shown in D. (D) 40% development; leg segments are defined externally. An additional circumferential ring of high *dpp* expression has appeared proximal to the first ring; both rings are located intrasegmentally, in the presumptive tarsus and tibia. Scale bars, 10 μm.

RESULTS

dpp expression during grasshopper development

In fly embryos, *dpp* is initially expressed in a D/V gradient that resolves into two longitudinal stripes on each side of the embryo, one along the dorsal edge of the embryo and one more ventrolaterally, which runs through the dorsal side of the disc primordia during the allocation phase (Cohen et al., 1993; Goto

and Hayashi, 1997). Later, during the patterning phase of leg development, *dpp* is expressed at high levels in an anterior dorsal sector and at low levels in an anterior ventral sector of leg imaginal discs (Masucci et al., 1990; Theisen et al., 1996). We used an *S. americana dpp* cDNA to identify *dpp* transcripts in grasshopper embryos. The coding region of the *S. americana dpp* cDNA shows 83.5% amino acid similarity to *Drosophila dpp* and probes derived from this cDNA hybridize to a single transcript on northern blots containing poly(A)$^+$ RNA from grasshopper embryos (Newfeld and Gelbart 1995).

In grasshoppers, we find that *dpp* expression is highly dynamic. We see parallels to both the dorsal embryonic stripe and the dorsal leg stripe of *Drosophila*. However, not all aspects of fly *dpp* expression are shared with grasshoppers. In particular, we see no parallel to the lateral longitudinal stripe of *dpp* that is thought to play a role in allocating the imaginal discs in the early *Drosophila* embryo (Goto and Hayashi, 1997). Furthermore, several features of *Schistocerca dpp* expression in the developing leg appear to lack parallels in flies. Most notable is a set of segmentally reiterated stripes suggestive of a leg segmentation role for *dpp* in grasshoppers. Here we describe the *dpp* expression pattern during early, mid and late leg development, phases in which *dpp* appears to serve different functions.

dpp mRNA expression during early grasshopper leg development does not parallel *Drosophila* allocation phase expression

At the earliest accessible stages in grasshopper development (approx. 15-20% of development (D)), *dpp* is expressed in two partial stripes in each hemisegment, paralleling the D/V axis. One stripe lies roughly in the middle of the segment and the other lies near the presumed intersegmental boundary (Fig. 1A,B). This pattern does not resemble any *dpp* expression pattern seen in the early *Drosophila* embryo. *dpp* is also expressed along the periphery of the germ anlage (Fig. 1C), a domain that corresponds to the most dorsal longitudinal stripe of *dpp* expression in the *Drosophila* embryo. This dorsal stripe persists throughout the stages we have examined here. There are no additional longitudinal stripes or modulation of expression along the D/V axis of the embryo, as seen in *Drosophila* embryos. Thus, early grasshopper *dpp* expression does not parallel *Drosophila dpp* allocation phase expression.

dpp expression during the earliest stages of grasshopper leg outgrowth resembles its expression during disc patterning in *Drosophila*

Prior to and immediately following the onset of leg outpocketing (approx. 25% D), *dpp* mRNA expression patterns change rapidly (Figs 1, 2). The intrasegmental stripes come to dominate and extend along both the dorsal and ventral sides of the presumptive leg fields (Fig. 1C). The ventral leg expression may correspond to the low level of *dpp* expression in the anterior ventral sector of the *Drosophila* leg imaginal disc, which has no known function. Shortly after outpocketing, the stripes become restricted to the dorsal side of the limb domain (Fig. 1D,E). At this stage, the leg bud expression pattern resembles that of the second and third instar leg imaginal discs of *D. melanogaster*, where *dpp* is expressed at high levels in a wedge across the anterior dorsal portion of the disc (Diaz-Benjumea et al., 1994; Jiang and Struhl, 1996; Theisen et al., 1996). Our data do not distinguish between two mechanisms that could result in this transition in grasshoppers: (1) ventral cells that are initially expressing *dpp* could shut-off *dpp* transcription while more dorsal cells initiate *dpp* transcription; or (2) the ventral midline tissue might be proliferating, pushing the ventrolateral tissue dorsally. However, the resemblance between *dpp* expression in grasshopper and fly limbs is transient.

dpp mRNA is transiently absent in the dorsal stripe and becomes expressed in circumferential rings in mid-stage leg development

In flies, once established, the dorsal stripe of *dpp* in the leg domain persists unmodulated throughout development (Massucci et al., 1990; also see below). In grasshoppers, however, the *dpp* expression pattern remains dynamic. Before 30% development, the dorsal leg stripe of *dpp* mRNA becomes undetectable (Fig. 2A). *dpp* mRNA is now detectable in two small clusters of cells located subterminally near each appendage tip, one cluster located anteriorly and the other posteriorly. In addition, there is a strong “armpit” node of expression (Fig. 2A), which likely derives from the intersegmental patches detected earlier (Fig. 1D,E). This expression domain has no apparent counterpart in *Drosophila*.

The limb patches transform into a nearly circumferential ring of *dpp* expression near the distal limb tip (Fig. 2B). This ring is initially incomplete ventrally (not shown), but eventually closes and clearly extends through both the anterior and posterior compartments of the leg. Expression of *dpp* in posterior compartments is unexpected. In *Drosophila*, *dpp* is repressed in the posterior leg compartment by *engrailed (en*) (Sanicola et al., 1995) whose expression along the A/P axis is conserved in *Schistocerca* (Patel et al., 1989). The dorsal leg stripe of grasshopper *dpp* expression returns, albeit weakly, and is marked by nodes of higher expression (Fig. 2C). These nodes appear to mark the positions along the P/D axis at which subsequent circumferential rings of *dpp* mRNA expression appear. The five primary leg segments are visible by 40% development, at which point it can be seen that the *dpp* expression rings are located roughly in the middle of the primary leg segments (Fig. 2D). We have not confirmed the presence of a ring in the coxal segment. In addition, each of the more distal leg segments has a fainter partial ring of expression, and there is a faint stripe along the ventral edge of the leg, which could correspond to the faint *dpp* expression in the ventral *wg* domain of the *Drosophila* leg imaginal disc.

The correspondence between rings of *dpp* expression and leg segments suggests that *dpp* plays a role in segmentation of the grasshopper leg. Importantly, the change in gene expression from dorsal stripe to intrasegmental rings precedes morphological segmentation (for the tarsal segment, compare Fig. 2C to 2D). Additional support for this role comes from the observation that *dpp* is also expressed in circumferential rings in the other appendages that become segmented – the antennae (Fig. 3A) and maxillary (Fig. 3B) and labial (not shown) palps – but not in appendages or appendage branches that remain unsegmented – the labrum, mandible and inner branches of maxillae (Fig. 3B) and labium (not shown). The only exception to this correspondence between rings of *dpp* expression and segmentation is that a ring of expression appears in the

pleuropodium (Fig. 3C), which is not considered a segmented appendage.

dpp mRNA expression in the late grasshopper leg

After limb segmentation is complete, *dpp* expression is no longer similar in different appendage types. In the legs, expression is restricted to a small region around the developing tarsal claws (Fig. 4) and in a partial stripe on the ventral edge of the femur (not shown). As the grasshopper metathoracic legs are morphologically modified for jumping, we looked carefully for differences in expression between the metathoracic legs and the more anterior legs. The only qualitative difference in *dpp* expression between T1-T2 and T3 legs occurs late in development, when *dpp* is expressed in a pair of spurs that develop at the distal tibial tip in T3 (Fig. 4B). Neither the tibial spurs nor the *dpp* expression occur in T1-T2 (Fig. 4A).

dpp expression in *Drosophila* pupal legs

The *dpp* expression pattern we observe in *Schistocerca* is markedly different than that reported for *Drosophila*. However, the *Drosophila dpp* expression pattern has only been reported through the early pupal stages (Masucci et al., 1990). To determine whether *Drosophila dpp* might be expressed in a segmentally reiterated fashion later in development, we analyzed *dpp* expression in pupal leg discs using UAS.GFP (Ito et al., 1997) driven by dpp-blink.Gal4 (Staehling-Hampton et al., 1994). In wild-type third instar larval leg imaginal discs, dpp-blink.Gal4 drives expression of UAS transgenes in the same spatial pattern seen using *dpp* in situ hybridization, though at slightly higher levels (e.g. Theisen et al., 1996). In early pupal legs, we observed GFP expression in a strong dorsal stripe with weaker ventral expression (up to approx. 6 hours after pupariation; Fig. 5A) as reported by Masucci et al. (1990) using *dpp* in situ hybridization. After the leg is fully everted and segments are clearly delineated (approx. 12 hours after pupariation), we observe two changes in the *dpp* expression pattern. The dorsal expression no longer forms a continuous stripe. There are periodic positions along both the dorsal and ventral sides that are lacking *dpp* expression, in regions that appear to correspond to the joints. Secondly, the expression levels become more equivalent on dorsal and ventral sides of the leg (Fig. 5B). This periodic expression does not result in segmental rings of expression and occurs much later in leg morphogenesis than the segmental rings we detect in the grasshopper legs. Just before eclosion, when leg remodeling is complete, *dpp* is also strongly expressed in two muscle fibers in the femur (Fig. 5C).

wg expression in the developing grasshopper leg

In contrast to the variation we have detected between grasshopper and fly *dpp* expression, the expression of the *wg* transcript is nearly invariant between grasshoppers (Fig. 6), beetles and flies (Baker, 1987, 1988; van den Heuvel et al., 1989; Nagy and Carroll, 1994). During grasshopper leg development, *wg* transcripts are detected in two to three rows of cells that extend from the ventral midline to the distal tip of the appendage (Fig. 6). The only minor variation detected is that the level of *wg* mRNA is not uniform within each stripe in the grasshopper. The distal portion of the limb has lower levels of *wg* transcript. This same pattern is seen in the antennal, gnathal and thoracic appendages, with the exception of the mandible (see Fig. 6A) in which the *wg* stripe disappears from the middle of the limb primordia, but remains present both ventrally and dorsally, as has been reported previously for *Tribolium* (Nagy and Carroll, 1994).

Dll expression in the developing grasshopper leg

Given the unexpected pattern of grasshopper *dpp* expression but conservation of *wg* expression, we next examined whether expression of Dll and Exd, two components of the *Drosophila* leg patterning network that function downstream of *wg* and *dpp*, also differ in their expression during the development of the grasshopper leg. In flies, *dll* expression is initiated in the center of the disc primordia during early embryogenesis (Cohen, 1990; Cohen et al., 1989). This expression persists throughout larval development. By mid-late third instar, a second more proximal domain of *dll* appears, which is regulated independently of *dpp* (Diaz-Benjumea et al., 1994). The distal domain covers the distal tibia and tarsus while the proximal domain is in the presumptive proximal femur/distal trochanter (Wu and Cohen, 1999). A brief report of the expression of Dll in developing grasshopper limbs has been published (Palopoli and Patel, 1998). We extend their observations by following Dll expression in developing grasshopper limbs from the initiation of Dll expression until leg segmentation is complete. The pattern of Dll expression in developing grasshopper and fly legs is very similar.

Dll expression was absent or only observed in the antennal primordia of embryos of the youngest embryos examined (15-20% D; not shown). Prior to limb outpocketing, about the same time that the intrasegmental *dpp* stripes come to dominate, Dll expression is initiated in bilaterally symmetrical spots in the maxillary, labial and thoracic segments (Fig. 7A). Comparison of the D/V extent of the *dpp* and Dll domains at this stage (Fig. 1C and 7A) suggests that *dpp* is not confined to the dorsal half of the Dll domain, but rather extends across most or all of it. The change in *dpp* to the more dorsal region (Fig. 1D) during early limb outgrowth would lead to its restriction to the dorsal half of the Dll domain slightly later in development.

As the limbs grow out from the body wall, nuclearly located Dll is initially expressed continuously along the P/D axis from the distal tip of the limb for approximately 60% of the axis length (Fig. 7B,C). Between 25 and 30% development, Dll expression begins to fade from a central portion of its domain (Fig. 7D), leading to a gap in Dll expression. As development proceeds, this gap expands (Fig. 7E-I). When the primary leg segments become morphologically visible, the terminal region of Dll extends from the distal tip of the tarsus proximally to the central part of the tibia, and a more proximal ring of expression encompasses the femur-trochanter joint. These expression patterns closely parallel Dll expression patterns found in crickets (Niwa et al., 1997), and resemble those in flies. In flies, however, the disjunct Dll domains may result from expression of Dll in a new region rather than from loss of expression in the middle of an initially contiguous domain (Diaz-Benjumea et al., 1994).

Exd expression in the developing grasshopper leg

In *Drosophila*, Exd protein patterns the coxa, trochanter and the adjacent body wall (Gonzalez-Crespo and Morata, 1996; Rauskolb and Irvine, 1999). In fly leg primordia, nuclear Exd and Dll expression domains overlap briefly in the early embryo

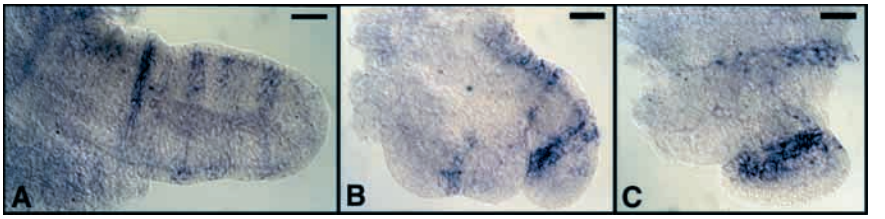

Fig. 3. *dpp* mRNA expression in other appendages of mid-stage grasshopper embryos (approx. 30% development). Note the rings in the (A) antenna (body wall attachment to left); (B) maxilla (body wall attachment at top); and (C) pleuropodium, an embryonic appendage on A1 (body wall attachment at top). These appendages are dissected from the embryo and shown in lateral view. Scale bars, 10 μm.

(Abu-Shaar and Mann 1998), remain adjacent until the end of the second instar, and then are separated during the third instar. During mid-late third instar, nuclear Exd and Dll overlap in the proximal ring of Dll. Using a monoclonal antibody developed against *Drosophila* Exd (Aspland and White, 1997), we examined the pattern of Exd expression and its relationship to Dll in grasshopper leg development from the first signs of leg differentiation until late leg segmentation.

In *Schistocerca*, nuclear Exd and Dll are expressed in adjacent but non-overlapping domains throughout most of the stages we examined (25%-40% D; Fig. 8). Early in development, Exd is nuclearly located in most cells of the embryo with the exception of small circles of cells expressing Dll, which will become the distal portions of the legs (Fig. 8A). As the leg bud grows, the Exd and Dll domains both expand and remain adjacent but non-overlapping (Fig. 8B). Following morphological segmentation of the legs, the boundary between nuclear Exd and the proximal ring of Dll-expressing cells lies in the trochanter. Late in limb development (40%) there is a small region of overlap (2-3 cells wide) between the proximal ring of Dll and the Exd domain (Fig. 8C), paralleling the overlap seen in mid-late third instar *Drosophila* leg discs.

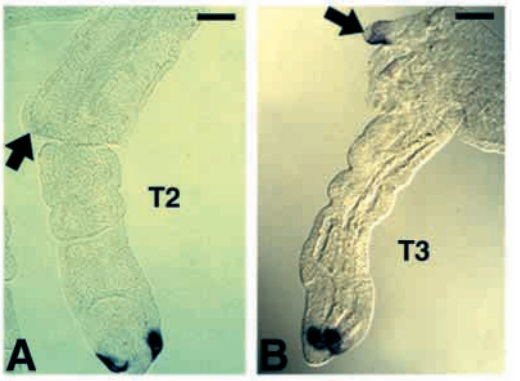

Fig. 4. *dpp* mRNA in the legs of grasshopper embryos (approx. 55% development). The legs have been dissected away from the body wall and are oriented with their attachment point towards the top, dorsal side to the left. (A) A mesothoracic leg showing *dpp* transcripts in two patches at the distal tip of the leg surrounding the presumptive claw. Arrow marks the tibia-tarsus boundary. (B) A metathoracic leg, in which *dpp* is also expressed in spurs at the distal end of the tibia. Scale bars, 10 μm.

DISCUSSION

We have analyzed molecular patterning during leg development in the grasshopper *Schistocerca americana*, in which legs develop directly, in order to evaluate the generality of the current *Drosophila* model for leg development. We find unexpected differences in *dpp* expression during the limb allocation stage. While grasshopper *wg* expression directly parallels *wg* expression in *Drosophila*, grasshopper *dpp* and *wg* never develop the ladder-like expression pattern seen during *Drosophila* leg allocation, which is hypothesized to be required for proper allocation of the limbs (Cohen et al., 1993; Goto and Hayashi, 1997). We also see only a transient resemblance to *Drosophila* in *dpp* expression during limb patterning. While *Drosophila dpp* is expressed in a continuous dorsal leg stripe throughout imaginal disc patterning, grasshopper *dpp* expression during leg development is highly dynamic. A dorsal leg stripe is present only at certain stages. In addition, a series of intrasegmental rings of *dpp* expression appear prior to leg segmentation in grasshoppers. This suggests that *dpp* plays a role in segmentation in grasshopper legs. Despite these early and late differences in *dpp* expression, Dll and Exd are expressed in similar patterns during the development of grasshopper and fly legs. In the absence of data on functional interactions among these genes during grasshopper limb development, the consequences of the major differences in *dpp* expression are not clear. However, the early divergence in expression of an upstream gene converging on similar expression of downstream targets

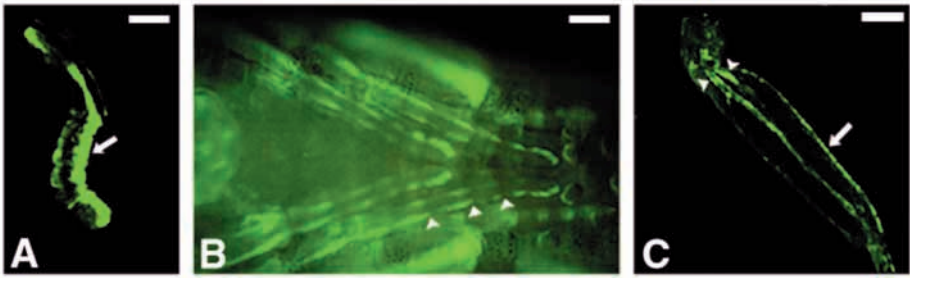

Fig. 5. UAS.GFP expression driven by a dpp-blink.Gal4 in *Drosophila* everted pupal legs. (A) Everted leg disc, approx. 6 hours after pupariation. Arrow points to the dorsal side of the leg. (B) Whole-mount preparations of everted pupal leg discs approx. 12 hours after pupariation. Fragmentation of the dorsal stripe is indicated by arrowheads. Anterior is to the left. (C) Femur of everted leg disc, just prior to eclosion. The arrow marks the dorsal epithelium; arrowheads demarcate the two *dpp* stained muscles. Scale bars, 10 μm.

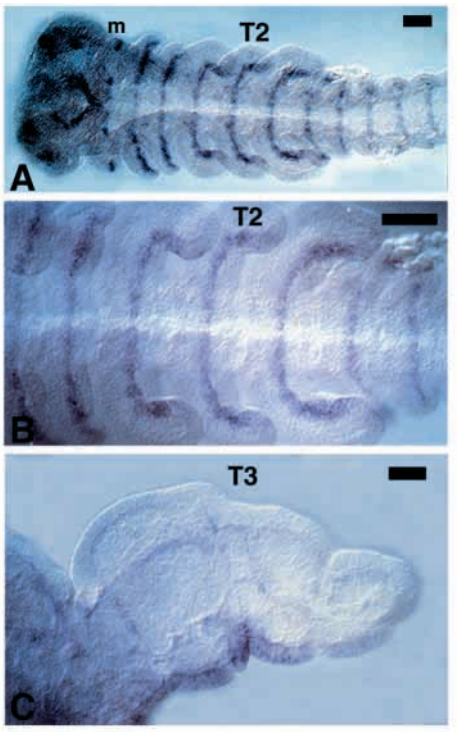

Fig. 6. *wg* mRNA expression in mid-stage grasshopper embryos. (A) 30% development. (B) Higher magnification of A. (C) Dissected metathoracic leg (45% development). The leg is oriented with ventral side down. Embryos in A and B are oriented ventral side up, with anterior to the left. m, mandibular segment; T2, second thoracic segment; T3, third thoracic segment. Scale bars, 10 μM.

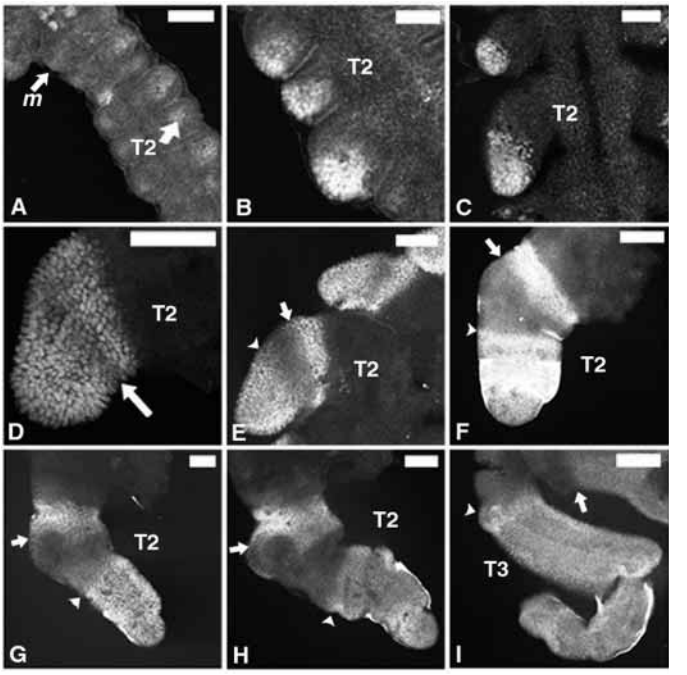

Fig. 7. Dll protein expression in grasshopper embryos. Whole-mount embryos (A-C,E) and dissected legs (D,F-I). (A) <25% development; this is the earliest stage at which Dll is detected in the thorax. Dll is detected in a small patch of cells within the gnathal (except for the mandible) and leg primordia (arrow). (B) 25% development and (C) <30% development; following limb outpocketing, Dll is expressed in a single domain in the distal limb. (D) <30% development; Dll is downregulated in a narrow central domain (arrow). (E-I) 30%-55% development; Dll becomes undetectable in this central domain (arrow and arrowhead in each panel), which expands as the leg grows. Following external segmentation (G-I), the distal Dll domain covers the tarsus and distal tibia and the narrower proximal domain spans the femur-trochanter joint. Scale bars, 10 μm. m, mandibular segment; T2, second thoracic segment; T3, third thoracic segment.

parallels the morphological differences between developing grasshopper and fly limbs.

Allocation and early patterning are evolutionary divergent in flies

In *Drosophila*, the early embryonic D/V gradient of *dpp* expression resolves into two longitudinal stripes, a lateral and a dorsal one, at the time of leg allocation. The lateral *dpp* domain marks the position of the imaginal disc and the dorsal edge of the circular disc of *dll* expression (Cohen et al., 1993). The appearance of the lateral longitudinal stripe of *dpp* in *Drosophila* is concordant with the transformation of the Dll domain from a linear stripe to a circular cluster of cells. This, combined with the observation that the Dll domain forms a stripe extending to the dorsal edge of the embryo in *dpp* mutants, led Goto and Hayashi (1997) to argue that the expression of *dpp* on the dorsal edge of the limb primordia during limb allocation is essential for expression of dll and proper allocation of imaginal discs in *Drosophila*. In grasshoppers, no lateral longitudinal stripe is observed. Instead, early in embryogenesis *dpp* is expressed in pairs of segmentally reiterated stripes that run perpendicular to the dorsal longitudinal stripe. Despite these differences, Dll is expressed in a circular disc, not expanded along the D/V axis.

These data are consistent with several possible scenarios for the evolution of limb allocation mechanisms. First, given the differences in early *dpp* expression, Dll expression and limb allocation might be regulated along the D/V axis by different mechanisms in the two species. Second, it might be possible to extract from the early grasshopper *dpp*/Dll expression patterns enough similarity to the *Drosophila* expression pattern to argue for conservation of the mechanism proposed by Goto

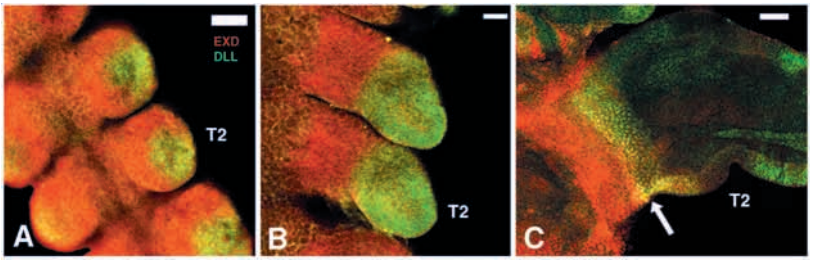

Fig. 8. Grasshopper legs double-labeled for Exd and Dll. Anterior is up, ventral is in the plane of the picture. Nuclearly localized Dll (green) and Exd (red) are expressed in adjacent but non-overlapping domains at (A) <25%development and (B) <30% development. (C) By 40% development, Dll and Exd overlap in a 3-4 cell wide band on the proximal edge of the proximal DLL domain (yellow; arrow). The distal femur and proximal tibia express neither nuclear Exd nor Dll. Scale bars, 10 μm.

and Hayashi (1997). In their model, the critical feature is the expression of *dpp* on the dorsal edge of the Dll domain. The early segmentally reiterated *dpp* stripes in the grasshopper would cover the dorsal edge of the Dll domain. However, *dpp* and Dll expression overlap extensively in grasshoppers and the overlap appears to extend beyond the center of the early Dll domain, a result that is unexpected from the Goto and Hayashi model. This leads us to the final scenario, in which the current model for how *dpp* restricts Dll in the *Drosophila* embryo (Goto and Hayashi, 1997) might not apply in either species. Instead, an as yet to be identified set of interactions may be operating commonly in both species.

dpp expression has also been examined in early *Tribolium* embryos and, as in *Drosophila*, a D/V gradient of expression is detected in the cellular blastoderm (Sanchez-Salazar et al., 1996), a stage inaccessible in *Schistocerca*. Later in embryogenesis, *Tribolium* resembles *Schistocerca* in having only a single longitudinal band of *dpp*-expressing cells, which is along the dorsal margin of the germband (Sanchez-Salazar et al., 1996). Presence of the dorsal longitudinal stripe in a grasshopper, a beetle and a fly suggests that this domain of *dpp* expression is evolutionarily old. By contrast, the lateral longitudinal stripe found in *Drosophila* is parsimoniously inferred to be a new feature that originated following the divergence of flies and beetles. Examination of *dpp* expression in additional taxa would reveal whether the appearance of the lateral longitudinal stripe is correlated with the evolution of leg imaginal discs in the higher Diptera. This would provide additional evidence on the evolutionary significance of this domain.

Although we cannot be sure without additional expression and functional data, the absence of the second longitudinal *dpp* stripe suggests that *Schistocerca* and *Tribolium* D/V axes, including the positioning of legs, are patterned via different genetic interactions than those functioning in *Drosophila*. Maxton-Kuchenmeister et al. (1999) arrive at a similar conclusion from their analysis of *Tribolium Toll*, another gene required to pattern the embryonic D/V axis in *Drosophila*. Interestingly, the *Drosophila* A/P axis is also patterned by a gradient, initially dependent on the graded distribution of the transcription factor Bicoid (Driever and Nusslein-Volhard, 1988). Despite indirect evidence for its existence in *Tribolium* (Wolff et al., 1998), *bicoid* has not been identified outside of dipteran insects. It is possible that the gradient mechanisms by which *Drosophila* imparts positional information along both its embryonic axes are not common to most insects. In the case of the A/P axis, the gradient may rely on a "new" gene, *bicoid*, whereas in the case of the D/V axis, *dpp* may have been co-opted for a new function.

Later leg patterning is more conservative

If the *Drosophila* model of leg disc patterning applied to *Schistocerca* leg patterning, grasshopper legs would express *wg* and *dpp* in stripes along the anteroventral and anterodorsal side of the leg respectively, Dll in a distal domain extending from the tibia to the distal tip and in a more proximal domain at the femur/trochanter boundary, and Exd in a more proximal circumferential domain, extending proximally into the body wall and distally to the femur. These expression patterns are observed throughout *Drosophila* second and third larval instar and pupal imaginal discs. The predictions for the *wg*, Dll and Exd domains are born out through the developmental periods we examined in grasshoppers, suggesting that their roles in P/D axis patterning are conserved. The conservation of Dll and *wg* expression patterns was expected given their expression in other hexapods (Panganiban et al., 1994, 1995; Niwa et al., 1997; Palopoli and Patel, 1998; Nagy and Carroll, 1994).

Despite only transient similarity in *dpp* expression in flies and grasshoppers, it is possible that some aspects of *dpp*'s role in establishing the P/D axis are conserved. In *Drosophila*, removal of Dpp signaling prior to the second larval instar results in loss of Dll expression while later removal of Dpp does not affect Dll expression (Lecuit and Cohen, 1997). Therefore, Dpp is thought to be required for the initiation but not maintenance of *dll* transcription in the leg imaginal disc (Lecuit and Cohen 1997). In grasshoppers, *dpp* expression is limited to an anterodorsal stripe transiently around the time of limb outpocketing. If maintenance of Dll expression in grasshoppers is also independent of *dpp* then the later changes in *dpp* expression would not be expected to affect expression of Dll.

While many aspects of the *Drosophila* leg imaginal disc patterning model are consistent with data obtained from grasshoppers, the extension of *dpp* onto the ventral side of the presumptive leg early in grasshopper leg development is unexpected. In *Drosophila*, functional analyses reveal that Wg represses Dpp ventrally in the leg imaginal disc (Theisen et al., 1996). The ventral *dpp* expression in grasshoppers may reflect absence of this mutual antagonism between Wg and Dpp during early leg development. Alternatively, this ventral expression may be homologous to the low-level expression of *dpp* seen in the ventral portion of *Drosophila* leg discs during second and third instars, which occurs in the presence of high levels of Wg, and apparently plays no role in *dll* activation. In grasshoppers, the ventral extension of *dpp* prior to and at the time of Dll activation would lead to a change in the distribution of cells expected to receive high levels of both Wg and Dpp signaling, which jointly activate *dll* in *Drosophila* imaginal discs. Despite this, the grasshopper and fly Dll patterns are similar. Additional functional data are required to evaluate the significance of this initial high level of *dpp* expression in a ventral domain of grasshopper limbs.

dpp may play a role in leg segmentation in grasshoppers

Morphologically, leg segmentation is remarkably conserved within all insects. The only notable variation occurs in the number of subsegments in the tarsus. In spite of this lack of morphological variation, we have uncovered an underlying molecular difference in leg segmentation between *Schistocerca* and *Drosophila*. We find that *dpp* is expressed in segmental rings in *Schistocerca*, but not, to the limits of our detection, in *Drosophila*. We suggest that *dpp* plays a role in establishing the primary leg segments during grasshopper limb development. Our evidence for this is twofold. First, the appearance of these segmentally reiterated rings precedes morphological segmentation of the legs. Second, similar rings of expression occur in serial homologues of the legs that become segmented but not in most non-segmented appendages. While several other proteins have been reported to be expressed in circumferential rings in grasshoppers –

annulin, a transglutaminase (Singer et al., 1992), fasciclin IV, a novel integral membrane protein (Kolodkin et al., 1992) and alkaline phosphatase (Chang et al., 1993) – Dpp is the first secreted signaling molecule known to be expressed in a manner that suggests a role in the establishment of the primary limb segments. The observation of rings of *dpp* expression in developing *Tribolium* limbs (E. J. and L. N. unpublished) suggests that this *dpp* expression domain is likely ancestral for the large clade of insects descended from the common ancestor of the orthopteroid and holometabolous orders, and was lost somewhere along the lineage leading to flies.

Violation of A/P boundary restrictions

As noted, the expression of *dpp* suggests variation in the fundamental regulatory mechanisms that pattern the P/D axis of the leg. It also suggests a violation of the rules that pattern the A/P axis of the *Drosophila* leg. In flies, the transcription of *dpp* is restricted to the anterior compartment of both leg and wing discs by En which represses *dpp* transcription in the posterior compartment (Sanicola et al., 1995). In grasshoppers, this anterior restriction is broken by the appearance of subterminal spots followed by circumferential rings at 30% embryogenesis. While Patel et al. (1998) noted variation in the intensity of En expression along the P/D axis in *S. americana*, En was always expressed throughout the posterior compartment. Thus, the transcription of *dpp* in the posterior of grasshopper limbs reflects a change in its regulatory circuitry involved in leg patterning relative to flies.

Evolution of imaginal discs

Adult fly and grasshopper limbs are morphologically similar. Both are five-segmented structures that emerge from the body wall at a defined position on the D/V axis. However, their developmental modes are quite different. Grasshopper limbs outpocket directly from the embryonic body wall during embryogenesis while fly limbs proliferate and are patterned as invaginated imaginal discs. All of the parallels we have drawn between fly and grasshopper limb development are at stages following the allocation of discs in flies. The main early difference we have identified is in *dpp*, a key upstream regulatory molecule in flies. This argues that the origin of leg imaginal discs in flies required alterations early in development, possibly affecting the mechanisms by which limbs are allocated in the early embryo. However, extensive later similarities in Dll and Exd indicate that these early differences eventually result in the activation of conserved patterning modules. Since Dll and Exd are both transcription factors, which function cell-autonomously, their function would not be expected to be affected by the differences in tissue architecture between imaginal discs and direct-developing legs. It is therefore perhaps not surprising that we have identified conservation at this level. While it is not straightforward to compare stages between grasshopper and fly limbs, the persistence of gene expression patterns in flies that characterize only a brief period in grasshopper limb development suggests that the early stages of grasshopper limb development have been expanded or slowed down to fill a much greater proportion of fly limb development. Such heterochronies are thought to be a common mechanism underlying morphological evolution.

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