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# Patterning of the branched head appendages in *Schistocerca americana* and *Tribolium castaneum*

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**SUMMARY** Much of our understanding of arthropod limb development comes from studies on the leg imaginal disc of *Drosophila melanogaster*. The fly limb is a relatively simple unbranched (uniramous) structure extending out from the body wall. The molecular basis for this outgrowth involves the overlap of two signaling molecules, Decapentaplegic (Dpp) and Wingless (Wg), to create a single domain of distal outgrowth, clearly depicted by the expression of the *Distal-less* gene (*Dll*). The expression of *wg* and *dpp* during the development of other arthropod thoracic limbs indicates that these pathways might be conserved across arthropods for uniramous limb development. The appendages of crustaceans and the gnathal appendages of insects, however, exhibit a diverse array of morphologies, ranging from those with no distal elements, such as the mandible, to appendages with multiple distal elements. Examples of the latter group include branched appendages or those that possess multiple lobes; such complex morphologies are seen for many crustacean limbs as well as the maxillary and labial appendages of many insects. It is unclear how, if at all, the known patterning genes for making a uniramous limb might be deployed to generate these diverse appendage forms. Experiments in *Drosophila* have shown that by forcing ectopic overlaps of Wg and Dpp signaling it is possible to generate artificially branched legs. To test whether naturally branched appendages form in a similar manner, we detailed the expression patterns of *wg*, *dpp*, and *Dll* in the development of the branched gnathal appendages of the grasshopper, *Schistocerca americana*, and the flour beetle, *Tribolium castaneum*. We find that the branches of the gnathal appendages are not specified through the redeployment of the Wg–Dpp system for distal outgrowth, but our comparative studies do suggest a role for Dpp in forming furrows between tissues.

## INTRODUCTION

Among arthropods there is incredible diversity in appendage morphology. In part, this diversity can be divided into three main appendage forms: uniramous, biramous, and phyllopodous (Manton 1977; Williams and Nagy 1996; Brusca and Brusca 2002). Uniramous appendages contain a single segmented distal outgrowth that extends from the body wall (Fig. 1A). Biramous appendages have two main distal outgrowths, the medial (ventral) endopod and lateral (dorsal) exopod (Fig. 1B). In addition to the two main branches, there may be multiple other simple branches termed exites and endites. The phyllopodous limb is a flattened limb that may also have many branches that emerge off of a main element (Fig. 1C). Though some of these branches are often referred to as endopods and exopods, the relationship between the elements of phyllopodous and biramous limbs is not clear. Extant crustaceans (depending on the species being examined) possess various combinations of uniramous, biramous, and phyllopodous appendages on their thorax and abdomen, whereas extant adult insects have only uniramous appendages on their thorax and have lost abdominal appendages altogether. The ancestral arthropod was aquatic and is thought to have possessed appendages with a branched morphology (Manton 1977). The insect uniramous condition may have arose out of the physical demands put upon terrestrial walking legs. Despite this apparent constraint on the walking appendages, insect gnathal appendages, which are serially homologous to the thoracic legs, have remained free to evolve very specialized structures to take advantage of different food sources and life strategies. For two of the gnathal appendages, the maxilla and labium, there have been some dramatic specializations (Snodgrass 1935); however, they are generally of a form that is common to *Schistocerca* and *Tribolium* (Fig. 1, D–F). The maxillae are made up of a palp, which bears resemblance to a segmented leg, and two basal endites, or branches, termed the galea and lacina (Snodgrass 1935). The labial appendages are similar to the maxillary

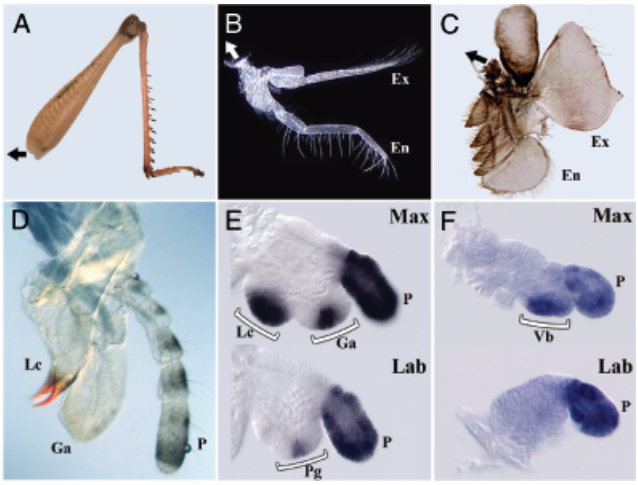


**Fig. 1.** (A) Adult grasshopper leg as an example of a typical uniramous insect appendage. (B) Biramous crustacean limb (*Mysidium columbiae*) showing the exopod (Ex) and endopod (En). (C) Phyllopodous crustacean limb (*Triops longicaudatus*) with a multibranched morphology. Arrows in A–C point toward the body wall attachment. (D) Branched maxilla of a grasshopper hatchling showing the lacina (Lc), galea (Ga), and palp (P). (E) *Schistocerca* maxilla (Max) and labium (Lab) stained for Dll protein showing three Dll domains in the maxilla, corresponding to the lacina, galea, and palp, and two Dll domains in the labium, corresponding to the paraglossa (Pg) and palp. (F) *Tribolium* maxilla and labium stained for Dll protein showing the two *Dll* domains of the maxilla, corresponding to the ventral branch (Vb) and the palp and the single *Dll* domain in the labial palp.

appendages and possess a palp and ventral branches, termed the glossa and paraglossa (Snodgrass 1935). In each case the branches are movable elements with unique muscle attachments to the base of the appendage. These branched structures may then resemble the branched appendages of the ancestor common to insects and crustaceans.

The molecular understanding of arthropod appendage patterning and development comes largely from work done in *Drosophila*. Wingless (Wg) signaling activates *buttonhead* (*btd*) in stage 10 and *Distal-less* (*Dll*) in early stage 11, which specify the limb primordia (Estella et al. 2003; Kubota et al. 2003). The precise position of the limb primordia, as indicated by *Dll* expression, along the mediolateral stripe of *wg* expression is determined by repression ventrally from receptor tyrosine kinase EGFR signaling and dorsally by Decapentaplegic (Dpp) (Goto and Hayashi 1997). Shortly after this initiation phase, *dpp* and EGFR are activated within the limb primordia where they are thought to play a role in setting up the subdivision of the appendage into proximal (*escargot*-expressing) and distal (*Dll*-expressing) precursor cells (Kubota et al. 2003). The distinction between these two phases is borne out on the *Dll* enhancer. Furthermore, during specification, *Dll* activation is controlled by different regulatory regions in the head and thorax, whereas a separate 933-bp enhancer is able to drive the later embryonic expression of *Dll*, when both Dpp and Wg are required, in both the head and thorax regions (Vachon et al. 1992).

In the leg imaginal disc, *wg* is expressed in a ventral quadrant and *dpp* is expressed in a stripe through the dorsoventral axis, with higher expression dorsally (Basler and Struhl 1994). The high level of Dpp in the dorsal part of the disc and Wg in the ventral part, together, are required to generate the single domain of distal outgrowth and *Dll* in the center of the disc, where their signaling domains overlap (Lecuit and Cohen 1997). Dll specifies "distal" fate and represses "proximal" fate by preventing the nuclear localization of the homeodomain protein Extradenticle (Exd), setting up an important division between proximal and distal leg cells and the proximodistal (PD) axis of the leg (Abu-Shaar and Mann 1998). Wg and Dpp are required to maintain *Dll* expression in the first two larval instars, because the loss of either signal results in the loss of *Dll*, but are later dispensable (Galindo et al. 2002). In the eye–antennal disc, from which the maxilla derives, the onset of *Dll* expression in the maxilla primordia is coincident with the delayed juxtaposition of Wg and Dpp signaling that does not occur until pupation (unpublished data). Thus, the Wg+Dpp model for PD patterning appears to account for distal outgrowth for the head as well as thoracic appendages during larval and pupal development.

The leg and head imaginal discs of *Drosophila* are very specialized structures and are not characteristic, in terms of their development, of most insect groups or arthropods as a whole (Svacha 1992). In the development of a more typical arthropod, as in *Schistocerca* and *Tribolium*, appendages are direct outgrowths from the embryo body wall. Initial analysis of *dpp*, *wg*, and Dll expression in the beetle, cricket, and grasshopper indicates a conservation of the pathway whereby Wg+Dpp results in distal outgrowth and *Dll* expression in the leg, despite radically different modes of development (Jockusch et al. 2000; Niwa et al. 2000; Dearden and Akam 2001; Chang et al. 2002). Dll is in the tips of virtually all arthropod distal outgrowths (Panganiban et al. 1994). This includes endites and exites of crustacean limbs and the ventral branches of the insect maxilla and labium. *wg* is expressed in stripes that run ventrally across each body segment to the tips of the developing limbs in each of the above species (Nagy and Carroll 1994; Niwa et al. 2000; Dearden and Akam 2001). *dpp* has a somewhat complex expression pattern but during the outgrowth of the limb bud is expressed at the tip of the developing leg (Sanchez-salazar et al. 1996; Jockusch et al. 2000; Niwa et al. 2000). Thus, *wg* and *dpp* expression in the leg are temporally and spatially positioned to specify distal outgrowth and pattern the PD axis in the appendages of these other insect groups in a manner similar to the *Drosophila* imaginal disc (Jockusch et al. 2000).

Although the *Drosophila* paradigm appears to apply to the development of uniramous appendages in various insects, it

remains to be seen how, if at all, these genes will function in patterning branched appendages, such as those found on various crustacean segments and in insect mouthparts. Experiments in *Drosophila*, however, do suggest a specific model for how these branched morphologies might be created. For the *Drosophila* imaginal disc it was found that when new domains of overlap between Wg and Dpp signals were created, either by activation of the Wg pathway in the dorsal Dpp domain or the Dpp pathway in the ventral Wg domain, a new domain of Dll expression was generated, which could create an artificially branched leg (Campbell et al. 1993; Basler and Struhl 1994; Diaz-Benjumea et al. 1994). This leads to the following questions: are the natural branches that form in crustacean appendages and in the gnathal appendages of insects "ectopic" distal outgrowths akin to the artificial branches created in the *Drosophila* experiments? Further, how similar are primary and secondary outgrowths with respect to patterning? To answer these questions we examined the expression patterns of components of the Wg+Dpp system in the maxilla and labium of *Schistocerca* and *Tribolium* to see whether they are consistent with having a role in generating secondary outgrowths. It should be noted that our study does not test the capacity of Wg and Dpp to generate ectopic outgrowths in other insects but whether or not this system is at work in a natural situation. If natural branches are indeed redeployments of the distal outgrowth pathway, we would expect to see the components of the Wg and Dpp pathways at positions corresponding to the position of each emerging branch. As the branches of these gnathal appendages are added as ventral elements to the initial palp, we would specifically expect to see a redeployment of *dpp* on the ventral *wg*-expressing surface of the appendage.

## MATERIALS AND METHODS

### Cloning

*Schistocerca wg* was cloned by Dan DiPietro and Nipam H. Patel with degenerate primers WgG, 5′-ARTGYAARTGYCAYGGNATG; WgR, 5′-TANCCNCKNCCRCARCACAT and is the same as the published sequence (Friedrich and Benzer 2000). Polymerase chain reaction was carried out for 40 cycles of 95°C for 30 sec, 48°C for 1.5 min, 72°C for 1.5 min. *Schistocerca dpp* was a gift from S. Newfeld (Newfeld and Gelbart 1995). *Tribolium dpp* and *gbb* were a gift from J. Doctor (Sanchez-salazar et al. 1996; M. J. Pincus, M. T. Pletcher, S. Missios, and J. S. Doctor, unpublished data). *Tribolium Dll* and *wg* were both cloned using gene-specific primers from the sequences available in GenBank. The primers for *Dll* were TcDll F, 5′-ATGTCGGGGGAGGCGCACATCGG; TcDll R, 5′-CACTTTCTTCAGTATTAAACAGCTGGCC. The primers for *wg* were TcWg F, 5′-ATGAGGAAAGAACTGTTCGG; TcWg R, 5′-AATTCGGCACGAGTTTATTAATG. Polymerase chain reaction was carried out for three cycles of 95°C for 30 sec, 70°C for 1 min, 72°C for 1 min and 30 cycles of 95°C for 30 sec, 65°C for 1 min, 72°C for 1 min. For a 900-bp fragment of the *Drosophila dpp*, 3′ untranslated region was used for an in situ probe.

### In situ hybridization

In situ hybridization was done using digoxigenin-, fluorescein-, and biotin-labeled anti-sense riboprobes. *Schistocerca* embryos were dissected in 1 × phosphate-buffered saline and fixed for 20 min in fixation buffer (100 mM Hepes, pH 6.9, 2 mM $MgSO_4$, 1 mM EGTA) with 3.7% formaldehyde. *Tribolium* were fixed as previously described (Patel et al. 1994). After rehydration the embryos were fixed a second time for 20 min, treated with a detergent solution (1% SDS, 0.5% Tween-20, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl), and incubated in hybridization buffer (50% formamide, 5 × SSC, pH 4.5, 0.1% Tween-20, 0.3% SDS, 50 μg/ml heparin, 100 μg/ml ssDNA) at 65°C for several hours before incubating overnight in probe diluted into hybridization buffer. Embryos were washed multiple times in hybridization buffer at 65°C over 3 h and then brought to room temperautre and washed multiple times in TBST buffer (25 mM Tris-HCl, 1.36 M NaCl, 26.8 mM KCl, 1% Tween-20) over 3 h. Embryos were incubated overnight at 4°C in the appropriate antibody. For two-color in situ hybridization, the first alkaline phosphatase reaction was done using nitro blue tetra-zolium chloride (NBT)/5-bromo-4-chloro-3-indolyl phosphate-4-toluidine (BCIP). After the reaction and several TBST washes, the embryos were incubated in hybridization buffer at 65°C for 10 min, allowed to cool, and washed out with TBST and put into the second antibody solution overnight at 4°C. The second color reaction was done using Fast Red (Boehringer-Mannheim, Indianapolis, IN, USA) dissolved in alkaline phosphatase buffer (pH 8.2) and allowed to react overnight at room temperature.

### Immunohistochemistry

*Tribolium* and *Schistocerca* embryos were fixed and stained as previously described (Patel et al. 1989, 1994). Dll protein expression was detected with a cross-reactive rabbit polyclonal antibody (Panganiban et al. 1994). Ps1, a cross-reactive rabbit polyclonal antibody that was used to visualize the phosphorylated form of the Mad protein, was a gift from P. Ten Dijke (Persson et al. 1998). Fluorescent detection was done using secondary antibodies Alexafluor-488 and Alexafluor-546. Anti-Exd was a gift from Rob White (Aspland and White 1997). Anti-βgal was from Cappell (Aurora, OH, USA). Embryos were mounted in glycerol.

## RESULTS

### *Schistocerca* mouthparts

Developmentally, the *Schistocerca* gnathal segments are delayed relative to the thoracic segments (Patel et al. 1989). The maxilla is made of a palp and two branches, the lacina and the galea. The maxilla starts as a bud that is similar to the thoracic leg buds. The tip of the bud corresponds to the maxillary palp. By 27% of development, the ventral surface of the developing maxilla begins to bulge out (Fig. 2, A–C). By 30%, this bulge has become two separate lobes distinct from the

palp (Fig. 2, D–F). These lobes will gradually become clear and distinct elements, the lacina and galea, as the palp extends distally (Fig. 2, D–I). Dll is expressed at the tip of the maxillary appendage bud in the presumptive palp. A second domain of Dll is detected in the ventral bulge at 28% of development (Fig. 2A). As the two lobes form, the Dll domain is maintained in the ventral-most lobe, the lacina (Fig. 2, A, D, and G). *Dll* is later activated in the galea by 35% (Fig. 2G). *wg* is expressed along the ventral surface of every segment, extending to the tip of the appendages (see Fig. 5A). *wg* expression is downregulated in the branches at later stages of development, after the onset of *Dll* expression (data not shown). Starting around 21% of development, a lateral domain of *dpp* expression partially overlaps *wg* in the maxillary segment in the future appendage bud. Initially, *dpp* is expressed in the tip of the maxillary palp, and by 28% a second domain of *dpp* appears on the ventral surface (Fig. 2B). As the new *dpp* domain overlaps with the *wg* stripe, the Wg+Dpp model would suggest that the new *dpp* domain is responsible for a second activation of the distal outgrowth pathway and the Dll expression in the bulge. However, as the two lobes take form, the initial *dpp* domain can be seen to lie in the furrow between the future lacina and galea (Fig. 2, E and H). Further, when Dll does finally appear in the galea, there is no corresponding *dpp* domain. Because Dpp is a signaling molecule it can affect a region larger than its expression domain. To assay the extent of *dpp* signaling, we used the Ps1 antibody, which is able to recognize the phosphorylated form of the Smad protein, a key member of the transforming growth factor (TGF)-β signaling pathway that is phosphorylated in cells receiving high levels of *dpp* signaling (Persson et al. 1998; Tanimoto et al. 2000). Ps1 is initially in the tips of all the appendage buds in a domain slightly larger than the Dll domain (Fig. 2C; also see Fig. 5B). Staining with Ps1 shows that no phospho-Smad is present on the bulge (Fig. 2C) nor in either branch (lacina or galea) through later stages of development (Fig. 2, F and I).

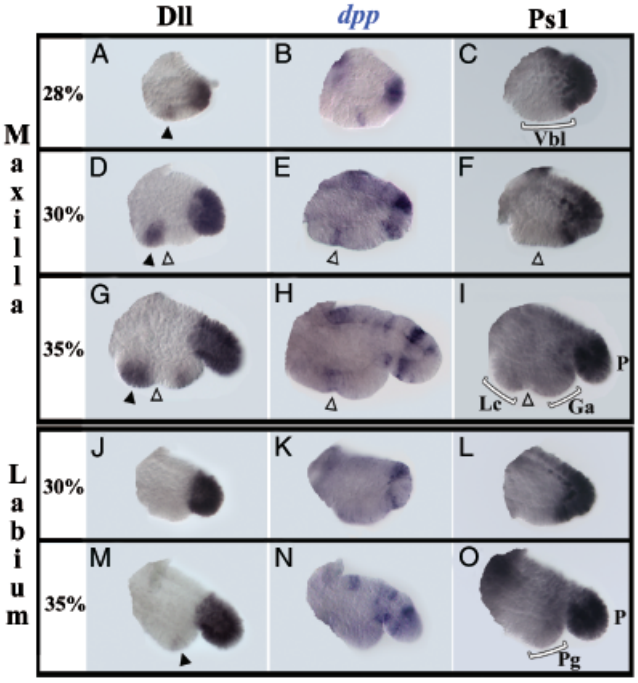


**Fig. 2.** *Schistocerca* maxillary and labial appendages stained for Dll protein, *dpp* mRNA, and phospho-Smad (Ps1). (A–I) Maxilla development; the maxilla is dissected out and oriented with ventral down and distal to the right. (A–C) At 28% of development, the ventral surface has a slight bulge (Vbl). (A) Dll is apparent in the middle of the ventral bulge (solid arrowhead). (B) *dpp* is also present on the ventral bulge. (C) There is no PS1 staining on the ventral surface where the Dll domain is. Ps1 is expressed in the palp and in the cells of the bulge just adjacent to the palp. (D–F) At 30% of development, there are two slight lobes forming on the ventral surface corresponding to the future lacina (Lc) and galea (Ga). The palp (P) is becoming a distinct element. Open arrowheads point to the furrow between the two maxillary branches. Solid arrowheads point toward the initial Dll domain. (D) Dll is clearly in the ventral-most lobe (lacina). (E) *dpp* is expressed in the furrow between the two lobes (open arrowhead). (F) Ps1 is expressed in the palp, is not expressed in the furrow nor the branch tips, but does maintain some expression at the edge of the forming galea that is closest to the palp. (G–I) At 35%, the branches and palp are more distinct elements. (G) Dll is expressed in the tips of both branches. (H) *dpp* remains in the furrow between the two branches. It has also formed several rings in the palp. (I) Ps1 remains expressed solely in the palp and lateral edge of the galea. (J–O) Development of a labial appendage, dissected and positioned the same as the maxilla in A–I. (J–L) At 30%, there is a ventral bulge apparent. (J) Dll is solely expressed in the palp. (K) *dpp* is expressed in a ring in the palp but not on the ventral surface of the bulge. (L) Ps1 is expressed in the palp and on the lateral edge of the ventral bulge. (M–O) At 35%, the labial branch, paraglossa (Pg), is distinct. (M) Dll is turned on in the ventral branch. (N) *dpp* is expressed in several rings in the palp but not in the ventral branch. (O) Ps1 remains expressed in the palp and in a weaker domain that covers that lateral edge of the branch.

The *Schistocerca* labial appendages also have a palp and two branches, the glossa and the paraglossa. As with the maxillae, the labial appendage possesses a palp but differs from the maxilla in that during most of embryogenesis it only forms a single lobe, the paraglossa, on the ventral surface. The paraglossa is a distinct element, but the glossae are not noticeable until a late stage of development. The glossae and paraglossae of the two appendages will eventually fuse medially to create the bottom lip of the grasshopper feeding apparatus. Dll is expressed first in the palp and later in the paraglossa around 35% (Figs. 1E and 2, J and M). *dpp* and Ps1 expression in the labial palp is similar to that in the maxillary palp; however, neither is expressed on the ventral surface in the paraglossa (Figs. 1E and 2, J–O).

## *Tribolium* mouthparts

The adult beetle maxilla has a palp and two branches, the galea and lacina, though a newly hatched larva has only a

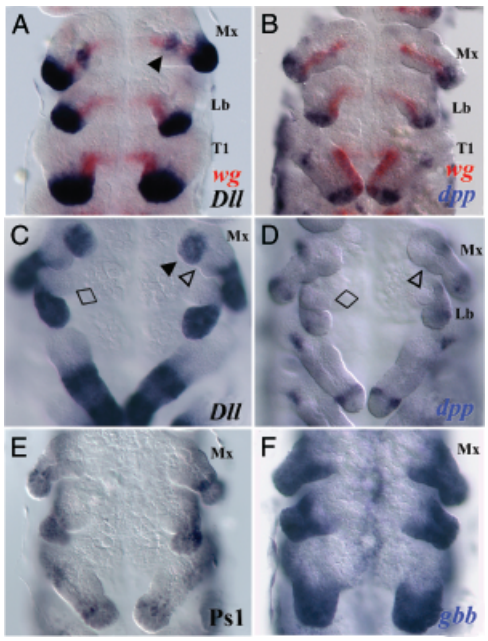


**Fig. 3.** *Tribolium* gnathal development. Views of the maxillary (Mx), labial (Lb), and first thoracic (T1) segments, with anterior to the left. Solid arrowheads show the *Dll* domain of the ventral branch. (A) Embryo stained for *wg* (red) and *Dll* (blue) showing the second *Dll* domain in the maxilla positioned on the ventral *wg* stripe (solid arrowhead). (B) A slightly older stage embryo than in A stained for *wg* (red) and *dpp* (blue) mRNA showing *dpp* in the palp tips but not on the ventral surface of the maxilla. (C) An older embryo stained for *Dll* mRNA. Notice that at this stage there are two lobes in the maxilla (open arrowhead points in between the two lobes) and a bulge in the labium (diamond); however, *Dll* only stains a single lobe in the maxilla (solid arrowhead). (D) *dpp* mRNA in an equivalent stage embryo as C; there are rings of dpp in the palps of the maxillae, labium, and legs, but there is no *dpp* expression on the ventral surface of either the maxillary or labial appendages. (E) An embryo stained for the phospho-Smad antibody, Ps1. Ps1 stains the palp of each appendage but not any other domains. (F) Tc *gbb* mRNA showing broad staining in each of the appendages.

single distinct visible branch (Rogers et al. 2002). *Dll* is first expressed in the tip, or palp, of the maxilla. As the appendage bud grows, the ventral surface develops a slight bulge that expresses *Dll* (Fig. 3A). This bulge gives rise to two lobes potentially representing the lacina and galea; however, whereas the ventral-most lobe continues to express Dll, the medial lobe does so only weakly before being apparently reabsorbed into the first branch at later stages (Figs. 1F and 3C). *wg* is expressed in ventral stripes across each segment (Fig. 3, A and B). *dpp* is expressed in the tips of the palps and eventually will give rise to a ring of expression (Fig. 3, B and D). *dpp* is not expressed on the ventral surface of the maxilla (Fig. 3, B and D). Likewise, Ps1 staining shows that phospho-Smad is present in the palps but not on the ventral surface (Fig. 3E). To explore the possibility that another TGF-β molecule might play a role in specifying the ventral branch of the maxilla, we examined the expression of the *Tribolium* homolog of the *Drosophila glass bottom boat* (*gbb*) gene, which signals with Dpp to properly pattern the wing imaginal disc (Ray and Wharton 2001). *Tribolium gbb* is expressed broadly in all the appendages. Its domain covers the region that will give rise to the branch, as well as the rest of the maxilla (Fig. 3F).

The labial appendage will eventually consist of a labial palp and a glossal lobe. The glossal lobes of the two labial appendages will fuse medially. During the phases of embryogenesis we were able to study, the palps are the only elements to express *Dll*. Expression of *dpp*, *Dll*, and Phospho-Smad in the palp is similar to that in the maxilla, but there are no secondary domains of these genes ventrally (Fig. 3, A–F).

## Exd expression

The observations described above suggest that Wg and Dpp do not act together to specify the *Dll* domains or distal outgrowths that form in the maxillary and labial branches (lacina, galea, and paraglossa). Thus, the patterning of these branches seems distinct from the patterning of the palp and legs. We examined Exd and Dll in the *Schistocerca* maxilla and labium to see whether the *Dll*-expressing branches could be defined as having the same PD properties as unbranched thoracic appendages. In the maxilla and labium, the Dll domain of the palp is juxtaposed to the Exd domain of the base, a relationship that is similar to that seen in the legs (Fig. 4, A–F) (Abzhanov and Kaufman 2000; Jockusch et al. 2000). Dll activation in the branch, however, is coincident with Exd staining, indicating that the palp and the branches form in different proximal/distal environments (Fig. 4B). To compare the insect gnathal appendage with a clearly biramous limb, we examined the localization of Exd in the biramous abdominal limb of the amphipod crustacean *Parhyale hawaiensis* (Fig. 4G) and found that the two primary branches, the endopod and exopod, both express *Dll* and are clear of Exd (Fig. 4, H and I). This indicates that the endopod and exopod are both distal elements, distinct from the proximal Exd domain, whereas the gnathal branches are not.

## Additional domains of *dpp* expression

That Ps1 did not stain in between the branches of the *Schistocerca* maxilla despite *dpp* expression prompted us to look for other *dpp* domains that did not correspond to regions of phospho-Smad. In *Schistocerca, dpp* has a striking expression domain at the lateral edges of the embryo between the segments, in the forming furrows (Fig. 5A). In the appendage-bearing segments, these domains lie in between the limb buds and remain there through much of development. *Tribolium* also have *dpp* expression in the forming furrows; however, it is more transient than in *Schistocerca* (Fig. 5C). In both animals Ps1 staining correlates with the domain at the tip of the limb bud but not the segmental furrow domain (Fig. 5B). We also

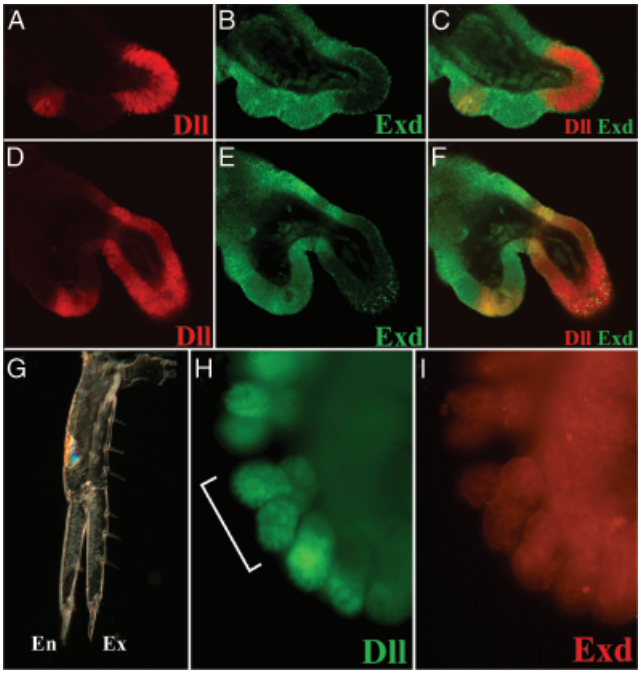


**Fig. 4.** Proximodistal properties of distal outgrowths. (A–C) At 30% of development, *Schistocerca* maxilla stained for Dll (red) and Exd (green) proteins. (A) At this stage Dll stains the palp and the lacina. (B) Exd is nuclear localized throughout the maxilla with the exception of the palp. (C) Overlap showing that Dll and Exd are exclusive in the palp but not in the developing branch. (D–F) At 40%, *Schistocerca* labial appendage stained for Dll (red) and Exd (green) proteins. (D) Dll stains the palp and the ventral branch. (E) Exd is nuclear localized throughout the labium with the exception of the palp. (F) Overlap showing that Dll and Exd overlap in the branch and the proximal part of the palp but are exclusive in the palp. (G) A biramous posterior limb from the amphipod crustacean, *Parhyale hawaiensis*. (H) Dll (green) is expressed in the two branches of a developing posterior limb (bracket) in *Parhyale*. (I) Exd (red) is not expressed in either of the two branches.

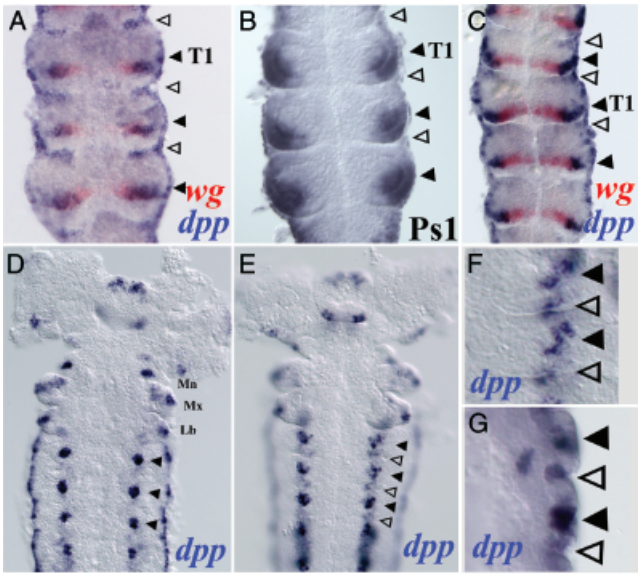


**Fig. 5.** *dpp* in the furrows. Solid arrowheads mark the limb fields, and open arrowheads mark the location of *dpp* in the furrows between the appendages. (A) *Schistocerca* embryo stained for *wg* (red) and *dpp* (blue) mRNA. (B) *Schistocerca* embryo stained for phospho-Smad. (C) *Tribolium* embryo stained for *wg* (red) and *dpp* (blue) mRNA. (D and E) *Drosophila* embryos stained for *dpp* mRNA. The embryos are dissected and flattened. (D) Stage 11 embryo showing the strong spots of expression that correspond to the forming limb primordia (solid arrowheads). (E) Slightly older stage 11 embryos than E showing the secondary *dpp* domains appearing in between the primordia spots (open arrowheads). Note that these domains lie within the forming morphological furrow. (F) First and second thoracic segments of a stage 12 *Drosophila* embryo magnified to show that the secondary *dpp* domain is positioned in the furrow. (G) Magnified view of the labial and first thoracic segments of a stage 12 embryo turned to the side to show the *dpp* domain in the furrows.

looked to see whether this expression domain in the furrow was conserved in flies. In the *Drosophila* embryo, *dpp* expression is often described as being in two longitudinal stripes. However, *dpp* is first expressed in spots that will correspond to the leg/wing primordia (Fig. 5D) (Goto and Hayashi 1997). *dpp* is then expressed in weaker spots that appear between the initial domains, in the embryonic segmental furrows (Fig. 5, D–G). Ps1 is broadly expressed across this region and seems to stain in the furrow as well (not shown). This may represent a greater conservation of the early *dpp* pattern between flies and other insects than previously thought.

During later leg development, rings form in the legs and gnathal palps of grasshoppers, beetles, and crickets (Sanchez-salazar et al. 1996; Jockusch et al. 2000; Niwa et al. 2000). In the grasshopper leg, the first ring arises from the tip domain by what appears to be the clearing of the distal-most cells of *dpp* (Jockusch et al. 2000). Multiple *dpp* rings will appear, at the segmental boundaries of the proximal leg as well as a few intrasegmental stripes (Fig. 6, A and B) (Jockusch et al. 2000). Phospho-Smad staining can be seen in the distal ring and the intrasegmental stripes but not in the intersegmental stripes (Fig. 6, C and D). Beetles form an initial ring of *dpp* expression at the limb and palp tips but do not form any other rings except for a dorsal patch of *dpp*, which does not form a complete ring of expression (Fig. 6E). This lack of *dpp* rings may be related to the fact that the embryonic *Tribolium* limb lacks the obvious furrows seen in *Schistocerca* and that *Tribolium* larvae form somewhat reduced legs, which will only be fully developed during pupation. We also reexamined the everting *Drosophila* leg to see whether there were any rings of *dpp* expression. Using a line that drives LacZ expression in a *dpp* pattern, from the BS3.0 fragment of the *dpp* 3′ regulatory region (Blackman et al. 1991), we found rings of expression in between the tarsal segments in the late pupal leg (Fig. 6F). This result differs from a previous study, which used a UAS.GFP driven by dpp-blink.Gal4 and found no evidence of pupal leg rings (Jockusch et al. 2000). The dpp-blink element is a 4-kb

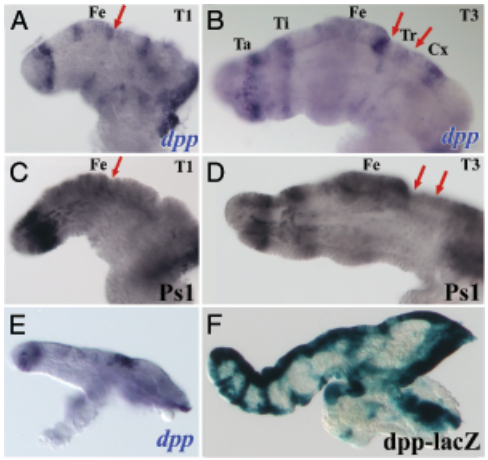


**Fig. 6.** *dpp* leg rings. Cx, coxa; Fe, femur; Ta, tarsus; Ti, tibia; Tr, trochanter. Red arrows indicate location of intersegmental rings of *dpp*. (A–D) Legs of *Schistocerca*. (A) The T1 leg shows rings of *dpp*: at the tarsi, the femoral–trochanter joint and the femoral–tibial joint. (B) A slightly older T3 leg showing both intersegmental stripes, such as the femoral–trochanter and trochanter–coxal joints, and intrasegmental stripes in the tibia and femur. (C) A T1 leg stained for the Ps1 antibody. Phospho-Smad is only present in the tarsal ring. (D) A T3 leg showing that Ps1 has ringed expression corresponding to the position of intrasegmental *dpp* stripes. (E) *Tribolium* leg stained for dpp; the only complete ring is in the tarsal segment. (F) βgal activity stain of a *Drosophila* everting leg disc from a dpp-lacZ line showing rings in between the tarsal segments.

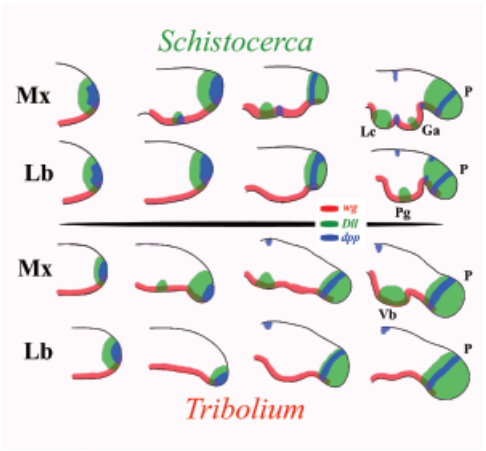


**Fig. 7.** Summary of the development of the maxilla (Mx) and labium (Lb) in *Schistocerca* and *Tribolium*. *wg* (blue), *dpp* (red), and Dll (green) are shown in the appendage bud. The buds are oriented with dorsal up and distal to the right and are ordered from youngest to oldest (left to right). Ga, galea; Lc, lacina; P, palp; Pg, paraglossa; Vb, ventral branch.

fragment of the 3′ Disk enhancer, whereas the BS3.0 fragment is a 12-kb fragment of the disk enhancer and includes the entire blink element. We were unable to confirm this result by in situ hybridization for *dpp* mRNA because of the difficulty of performing in situ hybridization on tissue at this stage.

## DISCUSSION

The Wg+Dpp system for specifying distal outgrowth and patterning the PD axis of uniramous legs appears to be conserved across insects. However, not all distal outgrowths are specified through the Wg+Dpp pathway. The prediction that the ventral branches of the maxilla and labium form through the redeployment of the Wg+Dpp pathway does not appear to hold true. We show that the pathway that activates *Dll* in the gnathal branches appears to do so in the absence of input from the canonical Dpp signaling pathway and in the presence of nuclear-localized Exd, a marker for proximal fate. We also found evidence of a role for *dpp* in forming ectodermal furrows between body segments and between leg segments.

### Gnathal branches differ from other distal outgrowths

When the *dpp* or *wg* pathways are manipulated in the *Drosophila* second instar discs such that a novel intersection of the two signals is created, a novel distal domain is also created (Campbell et al. 1993; Basler and Struhl 1994; Diaz-Benjumea et al. 1994). The new domain expresses Dll and can lead to the development of a bifurcated limb. This ability to create an ectopic outgrowth in this manner may be a unique property of the derived imaginal disc and not a general capacity of appendage fields in other arthropods. Regardless, it is conceivable that natural limb branches, such as in the maxilla and labium, are formed by the redeployment of *wg* and *dpp* such that secondary distal outgrowths are created. However, we found no additional domains of *dpp* to correspond to the onset of Dll expression in the developing branches (Fig. 7). There is one possible exception to this as *dpp* does intersect the *wg* domain on the ventral surface of the *Schistocerca* maxilla; however, this domain of *dpp* appears to have more to do with forming furrows between the two branches of the maxilla than with creating a distal outgrowth. At the onset of *Dll* expression in the ventral bulge of the grasshopper maxilla there is a domain of *dpp* expression, which, though temporally and spatially very close to the onset of *Dll*, actually seems to be positioned in cells neighboring the *Dll* cells. As development proceeds, *dpp* and *Dll* can be seen to occupy distinctly different domains. Because *dpp* is not needed to maintain distal fate during later imaginal disc development (Lecuit and Cohen 1997), it is possible that Dpp specifies distal fate in the branch during a brief window of time. However, we do not detect any Ps1 staining in the ventral maxilla (Ps1 staining accompanies the *Dll* expression in the palps and legs as well as the antennae and labrum). We suggest that the *dpp* domain that appears on the ventral surface of the grasshopper maxilla may play a role in segmenting the two lobes of the maxilla and not in the activation of *Dll* (see below). These lines of

evidence lend support to the notion that the ventral branches are fundamentally different from other distal outgrowths and form through a mechanism different from the Wg+Dpp model for distal outgrowth. Further support comes from an interesting mutant *Dll* allele in *Tribolium*. The *Sa-BQ* allele is a noncoding mutation in which *Dll* expression is initiated but lost everywhere except the later neural pattern and in the ventral branch of the maxilla (Beermann et al. 2001). It thus seems likely that the enhancer for activation of *Dll* in the branch is separable from that necessary for limb specification and outgrowth.

Additionally, we showed that Dll is activated on the ventral surface of the maxilla in a nuclear Exd-positive domain, whereas the Dll domain of the palps and limbs is absent of nuclear-localized Exd. This is similar to data from crickets where Exd is expressed in the ventral branches of the maxilla and labium (Abzhanov and Kaufman 2000). In *Drosophila*, Exd normally represses the activity of Wg and Dpp signaling in the proximal leg disc; however, when *dpp* is overexpressed at high levels, Wg and Dpp overcome this repression, activate Dll, and prevent the nuclear localization of Exd (Abu-Shaar and Mann 1998; Gonzalez-Crespo et al. 1998). Further, the artificially branched *Drosophila* legs, which are redeployments of the Wg+Dpp system, often possess multiple segments in an apparent attempt to make complete duplications (Campbell et al. 1993; Struhl and Basler 1993; Diaz-Benjumea et al. 1994), whereas gnathal branches and crustacean endites consist of a single segment (Snodgrass 1935; Schram 1986). We believe this supports a distinction between the outgrowths created by the Wg+Dpp system and endites, such as the gnathal branches, which are likely to be determined through a different system. A true biramous limb may then only be found in the crustaceans. We showed that in an amphipod crustacean biramous limb, both endopod and exopod express Dll in the absence of nuclear-localized Exd. The branchiopod crustaceans *Triops longicaudatus* and *Thamnocephalus platyurus* possess phyllopodous limbs that, though not easily homologized to the biramous limb, do show that ventral branches or endites express Dll in the presence of Exd whereas the endopod and exopod do so in the absence of Exd (Williams et al. 2002). Thus, the insect gnathal branches may be more analogous to crustacean endites and are patterned through a mechanism different from the Wg+Dpp system for primary limb outgrowths.

## Other roles for *dpp*

*dpp* has a very dynamic expression pattern throughout development. We found that some of the expression domains of *dpp* correlate with phospho-Smad expression, whereas others do not. There are several possible explanations: the levels of phosphorylated Smad are too low for us to detect with the Ps1 antibody, the *dpp* domains are not biologically relevant, or Dpp may signal through other Smad-independent pathways. There is growing evidence for TGF-β family members signaling through Smad-independent pathways in vertebrates (Derynck and Zhang 2003; Hassel et al. 2003). In grasshoppers, *dpp* forms multiple rings in the leg. The first ring of *dpp* is in the limb tip or presumptive tarsi, which is followed by rings in the femur, tibia, and at the femoral–trochanter and trochanter–coxal joints. Ps1 initially stains most of the limb bud, then restricts to a ring in the presumptive tarsi much like *dpp* at that stage, and later stains the intrasegmental rings of the femur and tibia. Ps1 is notably absent from the femoral–trochanter and trochanter–coxal joints as well as the furrow domain in between the body segments. Phospho-Smad is also absent from the ventral surface of the gnathal appendages of both beetles and grasshoppers. These regions of *dpp* expression that are absent of Ps1 staining do have one common feature: they are all domains that are going to physically partition two ectodermal domains through the formation of a furrow. The furrow domains of the body lie in between adjacent body segments, the leg rings lie between two leg segments, and the ventral domain in the grasshopper maxilla lies in between two lobes or branches. The case in the maxilla is of particular interest because neither the *Schistocerca* labium nor *Tribolium* maxilla has a ventral *dpp* domain, but during development they also generate only a single distinct lobe, whereas the grasshopper maxilla has ventral *dpp* expression and generates two clear ventral branches. Grasshoppers are considered a basal insect and possess a direct or holometabolous development similar to many crustaceans. Hemimetabolous insects such as beetles and flies are much more derived, possessing a specialized larval stage that interrupts the transition from embryo to adult. *Schistocerca* and many crustaceans must develop complete appendages during embryogenesis, whereas *Tribolium* and *Drosophila* hatch with reduced or separate specialized structures, forming the adult structures during larval or pupal stages. The loss of a *dpp* domain in the *Tribolium* maxilla may reflect this shift from holometabolous to hemimetabolous development. In direct developing *Schistocerca*, *dpp* is associated with the creation of a distinct galea and lacina during embryogenesis. *Tribolium* larvae possess a reduced maxilla with only a single prominent lobe, which only forms a distinct galea and lacina in the transition to the adult. The absence of a ventral *dpp* domain in the maxilla during embryogenesis correlates with this loss of distinct branches in the larvae. Thus, contrary to our expectations, it seems that *dpp* is not used for making branches by creating new PD axes but may play a role in creating invaginations between body segments, limb segments, and limb branches.

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## REFERENCES

Abu-Shaar, M., and Mann, R. S. 1998. Generation of multiple antagonistic domains along the proximodistal axis during *Drosophila* leg development. *Development* 125: 3821–3830.

Abzhanov, A., and Kaufman, T. C. 2000. Homologs of *Drosophila* appendage genes in the patterning of arthropod limbs. *Dev. Biol.* 227: 673–689.

Aspland, S. E., and White, R. A. 1997. Nucleocytoplasmic localisation of extradenticle protein is spatially regulated throughout development in *Drosophila*. *Development* 124: 741–747.

Basler, K., and Struhl, G. 1994. Compartment boundaries and the control of *Drosophila* limb pattern by hedgehog protein. *Nature* 368: 208–214.

Beermann, A., Jay, D., Beeman, R., Hulskamp, M., Tautz, D., and Jurgens, G. 2001. The Short antennae gene of *Tribolium* is required for limb development and encodes the orthologue of the *Drosophila* Distal-less protein. *Development* 128: 287–297.

Blackman, R. K., Sanicola, M., Raftery, L. A., Gillevet, T., and Gelbart, W. M. 1991. An extensive 3′ cis-regulatory region directs the imaginal disk expression of decapentaplegic, a member of the TGF-beta family in *Drosophila*. *Development* 111: 657–666.

Brusca, R. C., and Brusca, G. J. 2002. *Invertebrates*. Sinauer Associates, Sunderland, MA.

Campbell, G., Weaver, T., and Tomlinson, A. 1993. Axis specification in the developing *Drosophila* appendage: the role of wingless, decapentaplegic, and the homeobox gene aristaless. *Cell* 74: 1113–1123.

Chang, C. C., Dearden, P., and Akam, M. 2002. Germ line development in the grasshopper *Schistocerca gregaria*: vasa as a marker. *Dev. Biol.* 252: 100–118.

Dearden, P., and Akam, M. 2001. Early embryo patterning in the grasshopper, *Schistocerca gregaria*: wingless, decapentaplegic and caudal expression. *Development* 128: 3435–3444.

Derynck, R., and Zhang, Y. E. 2003. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* 425: 577–584.

Diaz-Benjumea, F. J., Cohen, B., and Cohen, S. M. 1994. Cell interaction between compartments establishes the proximal-distal axis of *Drosophila* legs. *Nature* 372: 175–179.

Estella, C., Rieckhof, G., Calleja, M., and Morata, G. 2003. The role of buttonhead and Sp1 in the development of the ventral imaginal discs of *Drosophila*. *Development* 130: 5929–5941.

Friedrich, M., and Benzer, S. 2000. Divergent decapentaplegic expression patterns in compound eye development and the evolution of insect metamorphosis. *J. Exp. Zool.* 288: 39–55.

Galindo, M. I., Bishop, S. A., Greig, S., and Couso, J. P. 2002. Leg patterning driven by proximal-distal interactions and EGFR signaling. *Science* 297: 256–259.

Gonzalez-Crespo, S., Abu-Shaar, M., Torres, M., Martinez, A. C., Mann, R. S., and Morata, G. 1998. Antagonism between extradenticle function and Hedgehog signalling in the developing limb. *Nature* 394: 196–200.

Goto, S., and Hayashi, S. 1997. Specification of the embryonic limb primordium by graded activity of Decapentaplegic. *Development* 124: 125–132.

Hassel, S., et al. 2003. Initiation of Smad-dependent and Smad-independent signaling via distinct BMP-receptor complexes. *J Bone Joint Surg. Am.* 85[A] (suppl. 3): 44–51.

Jockusch, E. L., Nulsen, C., Newfeld, S. J., and Nagy, L. M. 2000. 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. *Development* 127: 1617–1626.

Jockusch, E. L., Williams, T. A., and Nagy, L. M. 2004. The evolution of patterning of serially homologous appendages in insects. *Dev. Genes Evol.* 214: 324–338.

Kubota, K., Goto, S., and Hayashi, S. 2003. The role of Wg signaling in the patterning of embryonic leg primordium in *Drosophila*. *Dev. Biol.* 257: 117–126.

Lecuit, T., and Cohen, S. M. 1997. Proximal-distal axis formation in the *Drosophila* leg. *Nature* 388: 139–145.

Manton, S. M. 1977. *The Arthropoda: Habits, Functional Morphology, and Evolution*. Oxford University Press, Oxford.

Nagy, L. M., and Carroll, S. 1994. Conservation of wingless patterning functions in the short-germ embryos of *Tribolium castaneum*. *Nature* 367: 460–463.

Newfeld, S. J., and Gelbart, W. M. 1995. Identification of two *Drosophila* TGF-beta family members in the grasshopper *Schistocerca americana*. *J. Mol. Evol.* 41: 155–160.

Niwa, N., et al. 2000. Correlation of diversity of leg morphology in *Gryllus bimaculatus* (cricket) with divergence in dpp expression pattern during leg development. *Development* 127: 4373–4381.

Panganiban, G., Nagy, L., and Carroll, S. B. 1994. The role of the Distal-less gene in the development and evolution of insect limbs. *Curr. Biol.* 4: 671–675.

Patel, N. H., Condron, B. G., and Zinn, K. 1994. Pair-rule expression patterns of even-skipped are found in both short- and long-germ beetles. *Nature* 367: 429–434.

Patel, N. H., Kornberg, T. B., and Goodman, C. S. 1989. Expression of engrailed during segmentation in grasshopper and crayfish. *Development* 107: 201–212.

Persson, U., et al. 1998. The L45 loop in type I receptors for TGF-beta family members is a critical determinant in specifying Smad isoform activation. *FEBS Lett.* 434: 83–87.

Ray, R. P., and Wharton, K. A. 2001. Context-dependent relationships between the BMPs gbb and dpp during development of the *Drosophila* wing imaginal disk. *Development* 128: 3913–3925.

Rogers, B., Peterson, M., and Kaufman, T. 2002. The development and evolution of insect mouthparts as revealed by the expression patterns of gnathocephalic genes. *Evol. Dev.* 4: 96–110.

Sanchez-salazar, J., et al. 1996. The *Tribolium* decapentaplegic gene is similar in sequence, structure, and expression to the *Drosophila* Dpp gene. *Dev. Genes Evol.* 206: 237–246.

Schram, F. R. 1986. *Crustacea*. Oxford University Press, New York.

Snodgrass, R. E. 1935. *Principles of Insect Morphology*. McGraw Hill, New York.

Struhl, G., and Basler, K. 1993. Organizing activity of wingless protein in *Drosophila*. *Cell* 72: 527–540.

Svacha, P. 1992. What are and what are not imaginal discs: reevaluation of some basic comcepts (Insecta, Holometabola). *Dev. Biol.* 154: 101–117.

Tanimoto, H., Itoh, S., ten Dijke, P., and Tabata, T. 2000. Hedgehog creates a gradient of DPP activity in *Drosophila* wing imaginal discs. *Mol. Cell* 5: 59–71.

Vachon, G., Cohen, B., Pfeifle, C., McGuffin, M. E., Botas, J., and Cohen, S. M. 1992. Homeotic genes of the Bithorax complex repress limb development in the abdomen of the *Drosophila* embryo through the target gene Distal-less. *Cell* 71: 437–450.

Williams, T., and Nagy, L. 1996. Comparative limb development in insects and crustaceans. *Semin. Cell Dev. Biol.* 7: 615–628.

Williams, T. A., Nulsen, C., and Nagy, L. M. 2002. A complex role for distal-less in crustacean appendage development. *Dev. Biol.* 241: 302–312.