Correlation of diversity of leg morphology in *Gryllus bimaculatus* (cricket) with divergence in *dpp* expression pattern during leg development

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SUMMARY

Insects can be grouped into mainly two categories, holometabolous and hemimetabolous, according to the extent of their morphological change during metamorphosis. The three thoracic legs, for example, are known to develop through two overtly different pathways: holometabolous insects make legs through their imaginal discs, while hemimetabolous legs develop from their leg buds. Thus, how the molecular mechanisms of leg development differ from each other is an intriguing question. In the holometabolous long-germ insect, these mechanisms have been extensively studied using *Drosophila melanogaster*. However, little is known about the mechanism in the hemimetabolous insect. Thus, we studied leg development of the hemimetabolous short-germ insect, *Gryllus bimaculatus* (cricket), focusing on expression patterns of the three key signaling molecules, *hedgehog* (*hh*), *wingless* (*wg*) and *decapentaplegic* (*dpp*), which are essential during leg development in *Drosophila*. In *Gryllus* embryos, expression of *hh* is restricted in the posterior half of each leg bud, while *dpp* and *wg* are expressed in the dorsal and ventral sides of its anteroposterior (A/P) boundary, respectively. Their expression patterns are essentially comparable with those of the three genes in *Drosophila* leg imaginal discs, suggesting the existence of the common mechanism for leg pattern formation. However, we found that expression pattern of *dpp* was significantly divergent among *Gryllus*, *Schistocerca* (grasshopper) and *Drosophila* embryos, while expression patterns of *hh* and *wg* are conserved. Furthermore, the divergence was found between the pro/mesothoracic and metathoracic *Gryllus* leg buds. These observations imply that the divergence in the *dpp* expression pattern may correlate with diversity of leg morphology.

Key words: *Gryllus bimaculatus*, Cricket, Leg development, *hedgehog*, *wingless*, *decapentaplegic*

INTRODUCTION

More than a million insect species have been identified so far, displaying a staggering variety of adult morphologies. One of fundamental questions in biology is how such various morphologies evolved on the earth. One of the ways in which to answer this question is to determine the molecular mechanisms of development in various insects (Akam and Dawes, 1992; Patel, 1993, 1994; Akam, 1994, 1998). The insects can be grouped mainly in two categories, holometabolous and hemimetabolous, according to the extent of their morphological change during metamorphosis. In holometabolous insects, the larvae are usually quite unlike the adult, and the pupal stage is present between the last larval stage and the adult. However, in hemimetabolous insects, the larva hatches in a form that generally resembles the adult, except for its small size and lack of wings and genitalia. How these molecular mechanisms of development differ from each other is thus an intriguing question. Although the mechanism in *Drosophila melanogaster*, which belongs to holometabolous, has been extensively studied, little is known about the mechanism in the hemimetabolous insect.

In order to study development of the hemimetabolous short-germ insect, we have employed the two-spotted cricket, *Gryllus bimaculatus*, instead of the grasshopper, because we can easily rear an inbred line of the cricket and use their eggs at any time (Niwa et al., 1997), and because the cricket is widely used to study insect physiology and neurobiology (Engel and Hoy, 1999; Paydar et al., 1999; Wenzel and Hedwig, 1999, other references are cited therein). There are two major differences in the developmental process between the fly (Fig. 1A-C) and cricket (Fig. 1D,E):

(1) Long and short germ – the development of *Drosophila* is typical of the long-germ mode of insect development, in which the pattern of segments is established by the end of the blastoderm stage. Short-germ insects, by contrast, generate all

or most of their metameric pattern after the blastoderm stage by the sequential addition of segments during caudal elongation (Fig. 1D).

(2) Imaginal disc and bud formation – the fly becomes adult through the pupal stage, in which new adult organs develop from the imaginal discs (e.g. Fig. 1B,C), while the cricket becomes adult without any profound discontinuity. The rudiments of the legs, eyes and other most of adult structures are formed by budding formation and already present at hatching (e.g. Fig. 1D,E).

As a first step to answer the question, we focused on expression patterns of the three genes, *hedgehog* (*hh*), *wingless* (*wg*) and *decapentaplegic* (*dpp*), because these genes code key signaling molecules during development that are involved in pattern formation of various tissues including segments, legs, guts, eyes, etc. in *Drosophila* (Lawrence and Struhl, 1996) as well as in vertebrates (Hogan, 1999). In this study, we cloned cDNAs for the *Gryllus hh*, *wg* and *dpp* genes, and then observed their expression patterns during development. The three genes are expressed in segments, leg buds, the fore- and hindgut, and eyes of the *Gryllus* embryo. Here, we report results related to leg development as a typical case.

We found that expression patterns of *Gryllus hh* and *wg* in the leg bud were exactly the same in the three thoracic legs and principally resembled those observed in the leg imaginal discs of *Drosophila*. These results suggest the existence of a common mechanism for leg pattern formation. However, it was found that expression pattern of *Gryllus dpp* in the metathoracic leg bud became distinct in later stages from that in the pro- or mesothoracic leg buds, which become morphologically different legs from the metathoracic leg. Furthermore, we found that *Gryllus dpp* was expressed in the leg bud in a different way from *Drosophila dpp* in the leg imaginal disc. Recently, Jockusch et al. (2000) reported that in the grasshopper *Schistocerca americana*, the expression pattern of *dpp* differs radically from that of *Drosophila dpp*. Interestingly, we found that the pattern of *Schistocerca dpp* is also distinct from the *Gryllus* pattern. These observations imply that divergence in the expression pattern of *dpp* may be related to diversity of the leg morphology, rather than difference in the developmental mode.

MATERIALS AND METHODS

Animals

All nymphs and adults of *Gryllus bimaculatus* (*Gb*, two-spotted cricket) were reared as described previously (Niwa et al., 1997). Staging of the embryos was carried out according to the Niwa et al., 1997 (see the number in Fig. 1D,E).

Cloning of *Gbhh*, *Gbwg* and *Gbdpp*

A cDNA fragment was cloned by PCR with degenerate primers designed as follows:

5′-MGNTGYAARGARAARYTNAA-3′ (outside 5′ primer), 5′-ACCCARTCRTGGCCNGG-3′ (outside 3′ primer), 5′-GT-NATGAAYCARTGGCCNGG-3′ (inside 5′ primer) and 5′-TCRAANCCNGCYTCNACNGC-3′ (inside 3′ primer) for *Gbhh*; 5′-GARTGYAARTGYCAYGGNATG-3′ (5′ primer) and 5′-ANG-TRCARTKRCANCYYTC-3′ (3′ primer) for *Gbwg*; and 5′-GGAATTCCGAYTGGATHGTNGCNCC-3′ (5′ primer) and 5′-CCATCGATGGACRCARCANGCYTTNGGNAC-3′ (3′ primer) for *Gbdpp*.

To isolate longer cDNAs, a cDNA library constructed with mRNA isolated from three-day-old *Gb* embryos was screened using the corresponding cDNA labeled with [^{32}P]-dCTP. The DNA sequences are available from GenBank/DDBJ under the Accession number AB044709 for *Gbhh,* AB044713 for *Gbwg,* and AB044710 for *Gbdpp*.

Whole-mount in situ hybridization and immunostaining

Standard protocols for whole-mount in situ hybridization using a digoxigenin (DIG)-labeled antisense RNA probe (Wilkinson, 1992) or double probes (one DIG-labeled and one fluoroscein isothiocyanate (FITC)-labeled) (Dietrich et al., 1997) for chick embryos and immunohistochemistry for grasshopper embryos (Patel et al., 1989) were used for cricket embryos. A monoclonal antibody against the engrailed protein, Mab 4D9, was purchased from the Developmental Studies Hybridoma Bank (The University of Iowa, Iowa City, USA). A polyclonal antibody against the Distal-less protein which is a marker protein for the distal identity of the leg bud (Cohen et al., 1989) was kindly provided by Dr Sean Carroll. Stained embryos were sectioned as described previously (Sasaki and Hogan, 1993).

RESULTS

Cloning of *Gryllus* cDNAs for *hedgehog*, *wingless* and *decapentaplegic*

We cloned *Gryllus* cDNA homologs of *hedgehog* (*Gbhh*), *wingless* (*Gbwg*) and *decapentaplegic* (*Gbdpp*) by screening a cDNA library constructed with mRNAs isolated from three-day-old *Gryllus* embryos (see Methods). Homologies of the deduced amino acid sequences for HH and WG between *Gryllus* and *Drosophila melanogaster* (*Dm*) are 68% (N-terminal half, Tashiro et al., 1993) and 69% (Rijsewijk et al, 1987), respectively. A partial deduced amino acid sequence of *Gb*DPP exhibits higher homology to the corresponding fragment of *Dm*DPP (54%, Padgett et al, 1987), *Schistocerca americana* DPP (64%, Newfeld et al, 1995) and *Tribolium castaneum* (*Tc*) DPP (48%, Sanchez-Salazar et al, 1996) than to that of *Dm*60A (30%, Wharton et al, 1991; Doctor et al, 1992) and *Dm*SCW (27%, Arora et al, 1994), which belong to the TGFβ family. We also cloned a cDNA fragment encoding a partial amino acid sequence of *Gb*60A that may be the *Drosophila* ortholog of 60A, because of a high homology of 79% (Wharton et al, 1991; Doctor et al, 1992). Judging from the high homology of the amino acid sequences of the three proteins between *Gryllus* and *Drosophila*, and from their expression patterns in embryos (see below), we concluded that *Gbhh*, *Gbwg* and *Gbdpp* were the orthologs of *Dmhh*, *Dmwg* and *Dmdpp,* respectively.

Expression patterns during germ-band elongation (stages 4-5) and segmentation (7-10)

We performed whole-mount in situ hybridization with *Gryllus* embryos to observe expression patterns of the three genes. In stages 4-5, during which the embryonic rudiment lengthens as posterior segments are added (Fig. 1D), *Gbhh* and *Gbwg* were expressed as stripes in either side of each segment boundary, as observed in *Drosophila*, and in the most posterior regions (Fig. 2), as revealed by double stain (Fig. 2D). With increase in the number of the segmental stripes posteriorly from 6 (Fig. 2A,B) to 7 (Fig. 2C,D), *Gbdpp* was expressed along the lateral edges of the embryo parallel to the A/P axis (Fig. 3A, see also
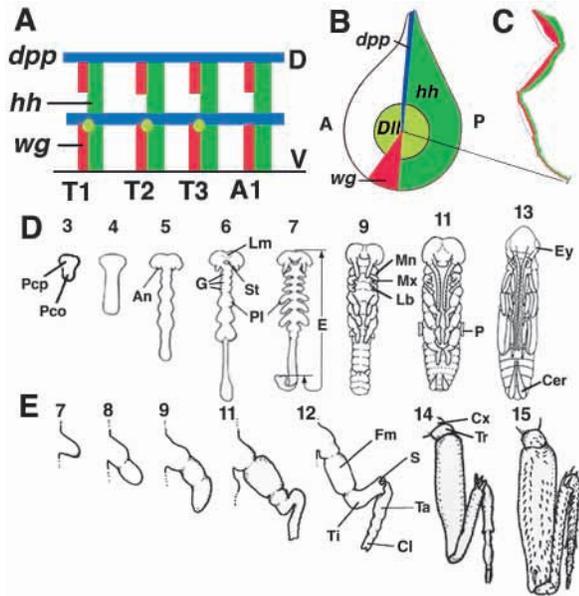
Fig. 1. Two different processes of insect leg development. (A-C) *Drosophila*. (D,E) *Gryllus* (A) In a holometabolous insect like a fly (*Drosophila*) the adult leg develops from the leg imaginal disc. At stage 12 of embryogenesis, *hh* (green) and *wg* (red) are expressed as stripes in the posterior and the anteroposterior (A/P) compartment boundary, respectively, while *dpp* (blue) is expressed in two longitudinal stripes in the lateral trunk and dorsalmost region. The primordia of the thoracic imaginal discs of the *Drosophila* embryo are allocated in response to signals from the *wg* gene product (see text for details). (B) The relationship between the third instar disc and the adult leg (C) is shown schematically. Distal-less (*Dll*) (light green) is expressed in the central domain where *wg* and *dpp* meet. (D) Illustrations showing *Gryllus* embryogenesis (ventral view). Numbers indicate developmental stages. In a hemimetabolous insect like a cricket, the adult leg develops from the limb bud formed in the embryo, as schematically illustrated. *Gryllus* is a short-germ insect: the protocephalon (Pcp) and protocorm (Pco) of presumptive anterior segments comprise most of the embryonic rudiment which lengthens as posterior segments are added during development. (E) Metathoracic leg formation. An, antenna; Cer, cercus; Cl, claw; Cx, coxa; Ey, eye; Fm, femur; G, gnathal segments; Lb, labrum; Lm, labium; Mn, mandible; Mx, maxilla; P, pleuropodium; Pl, prothoracic leg; S, tibial spur; St, stomodium; Ta, tarsus; Ti, tibia; Tr, trochanter.

Fig. 2. Expression patterns of *Gbhh* and *Gbwg* in *Gryllus* embryos at stages 4-5. All photos represent ventral views of the embryo with anterior to the left. In all panels, arrowheads marks the stripe 5 (the second thoracic segment and new stripe is indicated by a diamond). (A) Expression pattern of *Gbhh*: 5 segmental stripes and a new stripe are observed. (B) Expression pattern of *Gbwg* at stage 4. *wg* stripe initiates from the midline of the germ band and spreads dorsally. (C) Expression patterns of *Gbhh* and *Gbwg* at stage 4+: 6 segmental stripes and a new stripe are observed. (D) Double labeling reveals that the *Gbwg*-expressing cells (dark blue) reside directly anterior to the *Gbhh*-expressing cells (red). Scale bars, 100 µm.

expression domains became spots in each abdominal segment (Fig. 4G). Expression of *Gbdpp* was still detected in the cells of the putative dorsal ectoderm (Fig. 4K).

Expression patterns during leg-bud formation (stages 6-10)

The limb buds are formed in the three thoracic segments at stage 6 (Fig. 1D). These grow longer at stages 7-8 (3-4 day) (Fig. 1E) and become folded and grooved. The limb bud will become segmented by stages 9-10 (5-6 day) (Fig. 1E). In stages 7-10, *Gbhh* was expressed in the posterior region along the entire proximodistal (P/D) axis (Fig. 5A-D). This expression pattern coincided with the EN-expressing region (Niwa et al., 1997). *Gbhh* was expressed in posterior two third of the leg circumference, as shown in a transverse section of the leg bud (Fig. 5D). However, *Gbwg* was expressed as a stripe on the ventral side of the A/P boundary along the entire P/D axis of the leg bud, as shown in Fig. 5A (double stain with *Gbhh*) and in Fig. 5E-H, which is confirmed with transverse sections of the leg bud (Fig. 5H).

Expression of *Gbdpp* in the leg bud was first detected at stage 6.5 as a stripe, extending along the dorsal side of the limb bud from the longitudinal expression of the embryo to the distal tip of the limb bud continuously (data not shown). By stage 7, a faint *Gbdpp* stripe was observed along the dorsal side of the limb bud (Fig. 3B), which is then restricted to the dorsal side of the most distal tip of the limb bud (Fig. 3C). In stage 7, *Gryllus* Distal-less protein (*Gb*DLL) was detected with the specific antibody in the distal tip of the limb bud (Fig. 5M). At stage 9, *Gbdpp* was expressed as four spots

in Fig. 7A). Their expression patterns observed in earlier stages than 4 will be described elsewhere.

At stage 7, in which the embryos are fully segmented, *Gbhh* was expressed in the posterior region of each body segment (Fig. 4A,B). This expression domain coincides with the region where the engrailed protein (EN) is detected (Niwa et al., 1997). *Gbwg* was expressed in the ventral side of the anteroposterior (A/P) boundary of the body segments (Fig. 4E,F). Expression of *Gbdpp* remained at the dorsalmost region of each abdominal segment at this stage (Fig. 3B). At stage 8, *Gbdpp* was observed in the dorsal side of the distal tips of the elongating thoracic appendages (Fig. 3C). At stage 9, *Gbhh* was expressed continuously in the posterior compartment of each segment (Fig. 4C). *Gbwg* was expressed in the ventral side of the A/P boundary in the thoracic segments and elongating appendages (Fig. 4G). At this stage, the *Gbwg*
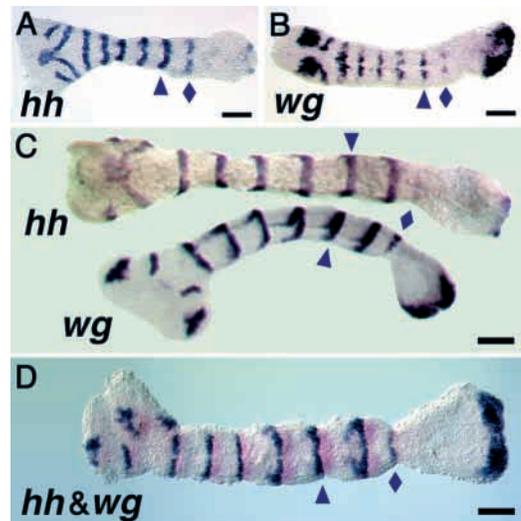
Fig. 3. Expression patterns of *Gbdpp* during *Gryllus* leg bud formation at stages 5, 7 and 8. (A) At stage 5, *Gbdpp* stripes are present along the lateral edge of the embryo, indicated by red arrowheads. A ventral view of the three thoracic segments and elongating abdominal segments is shown, where the anterior side is to the top. (B) A dorsal view of an embryo at stage 7, expression of *Gbdpp* is detected in the dorsal side of the distal tip of leg buds strongly and along the lateral edge of the embryo, indicated by red arrowheads. A weak stripe (arrow), continuing from the distal tip to the dorsal expression, is observed on the dorsal side of the leg bud. Region-specific expansions along the lateral edge of the embryo are observed on the abdominal segments (black arrowheads). (C) A dorsal view of an embryo at stage 8, the leg bud has a medial constriction and is divided into two presumptive segments, corresponding to the coxopodite and telopodite. Expression of *Gbdpp* is detected in the dorsal side of the distal tips of the elongating thoracic leg buds and in the dorsal side of the anterior-posterior boundary in each abdominal segment. T2, the mesothoracic segment. Scale bars, 100 µm.
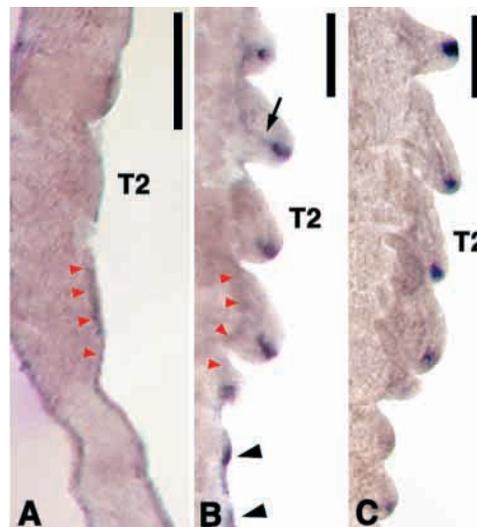
along the P/D axis in the dorsal side of the A/P boundary, although the most distal expression is not detected (Fig. 5J). At stage 10, the four spots of *Gbdpp* expression transformed into a nearly circumferential ring near the distal limb tips (data not shown). This ring is initially incomplete ventrally, but eventually closes and clearly extends through both anterior and posterior compartments of the leg. Other domains of *dpp* expression in more proximal segments still remained in patches at this stage.
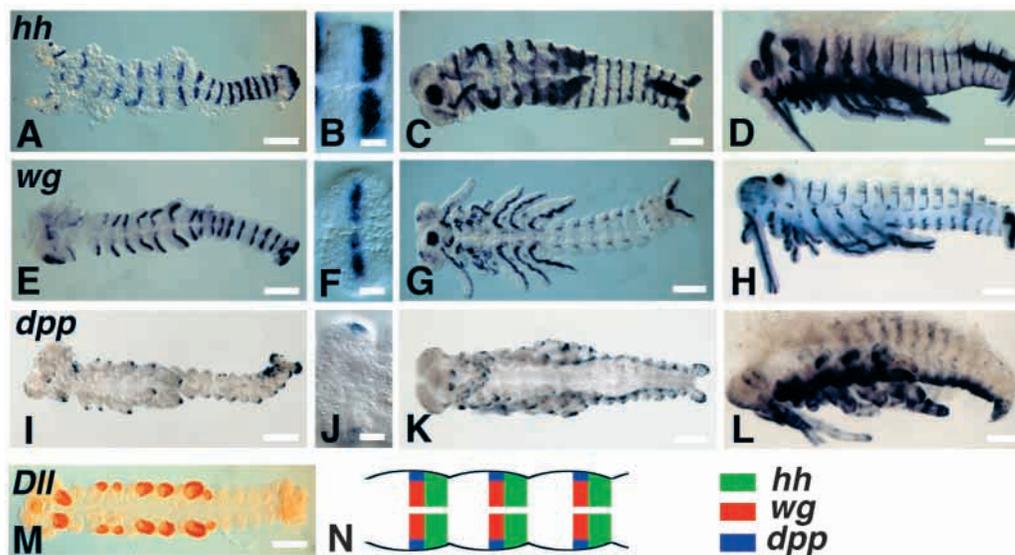
Expression patterns during leg-bud elongation after katatrepsis (stages 11-12)

In stage 11 (6-7 day) (Fig. 1D), in which the embryo moves around the posterior pole from dorsal to ventral side of the egg (katatrepsis), although signals of *Gbhh* in the posterior compartment of the abdominal segments became

narrow and weak, those in the lateral-dorsal regions of the thoracic segments became intense in addition to the signals in fore- and hind-guts (Fig. 4D). Expression of *Gbwg* in each segment is localized in the regions where trachea cells differentiate (Fig. 4H). The expression of *Gbdpp* also became intense as spots in the lateral regions of the thoracic segments (Fig. 4L).

The major leg segments are established at stages 11-12 (6-8 day) (Fig. 1E). In the mesothoracic limb bud, expression patterns of *Gbdpp* became five circumferential rings and two ventral spots (Fig. 5K), as schematically illustrated in Fig. 5N. The five *dpp* expression rings were located roughly in the middle of the primary leg segments of the tarsus, tibia and femur (Fig. 5K). In addition, three expression rings appeared in the presumptive three tarsus segments, prior to morphological change (Fig. 5K).

Fig. 4. Expression patterns of *Gbhh*, *Gbwg* and *Gbdpp* in *Gryllus* embryos at stages 7, 9 and 11. Segment-specific *Gbhh* stripes appear in the ventral posterior region of each segment at stages 7 (A,B), 9 (C) and 11(D). Segment-specific *Gbwg* stripes appear in the ventral anterior-posterior boundary of each segment at stage 7 (E,F), The *Gbwg* stripes changes to spots in the ventral side of the body and in the ventral side of the A/P boundary of the appendages at stage 9 (G). Expression of *Gbwg* was observed in the lateral side (H). *Gbdpp* was detected at the distal tips of the developing appendages and at the lateral edges of each abdominal

segment, which may correspond to the dorsal most cells of the segment at stage 7 (I,J). Then, *Gbdpp* is expressed as spots in the dorsal side of the A/P boundary of the appendage (K). *Gbdpp* is expressed as spots in the lateral side (L). DLL protein was localized in the distal parts of the appendage (M). Schematic illustration of expression patterns in each abdominal segment (N). Scale bars, 200 µm in A,C,D,E,G,H,I,K-M; 30 µm in B,F,J.

Fig. 5. Expression patterns of *Gbhh*, *Gbwg* and *Gbdpp* in *Gryllus* leg buds at stage 7, 9 and 11. Expressions of *Gbhh* was observed in the posterior region of the mesothoracic leg bud at stages 7 (A) (a double stain for *Gbhh* (red) and *Gbwg* (dark blue) is indicated by an open arrow), 9 (B) and 11 (C). The anterior side is towards the top. A transverse section at stage 11 reveals that *Gbhh* is expressed in the posterior region (D). Expression of *Gbwg* was observed in the ventral side of the limb bud along the anteroposterior boundary at stages 7 (E), 9 (F) and 11 (G). The dorsal side is towards the top. A transverse section at stage 11 reveals that *Gbwg* is expressed in the ventral region (H). A spot expression of *Gbwg* appears at the dorsal side of the coxa rudiment. Expression of *Gbdpp* was observed at the dorsal tip of the limb bud at stage 7 (I), as four spots at the dorsal side of the limb bud along the anterior-posterior boundary at stage 9 (J), and then as seven transverse strips at the regions corresponding to the leg segment structures at stage 11 (K). The dorsal side is to the top. A transverse section of mesothoracic tibia at stage 11 reveals that *Gbdpp* is expressed in both dorsal and ventral region (L). DLL protein was detected at the distal region of the leg bud (M). Schematic representation of expression patterns of *Gbdpp* in the leg bud at stage 11 (N). Segmental expression patterns correspond to the leg structures. In a cross section of the middle of the tibia (O), *Gbhh* (green) is expressed in the posterior compartment, while *Gbdpp* (blue) is expressed in both dorsal and ventral regions. *Gbwg* (red) expression overlaps *Gbdpp* expression (blue) in the ventral side of the A/P boundary, as indicated by purple. Expression patterns of *Dmdpp* in the leg imaginal disc (P,Q) and pupa

(R). Schematic representation of expression patterns in the cross section of the pupal leg imaginal disc (S). *Dmhh* (green) is expressed in the posterior compartment, while *Dmdpp* (blue) is expressed in both dorsal and ventral sides of the A/P boundary. *Dmwg* (red) expression overlaps *Dmdpp* expression (blue) in the ventral side of the A/P boundary, as indicated by purple. Scale bars, 20 μm in A, E,I,,M; 50 μm in B,F,J; 100 μm in C,G,K; 40 μm in D,H,L. Cx, coxa; Fm, femur; Ta, tarsus; Ti, tibia, Tr, trochanter.
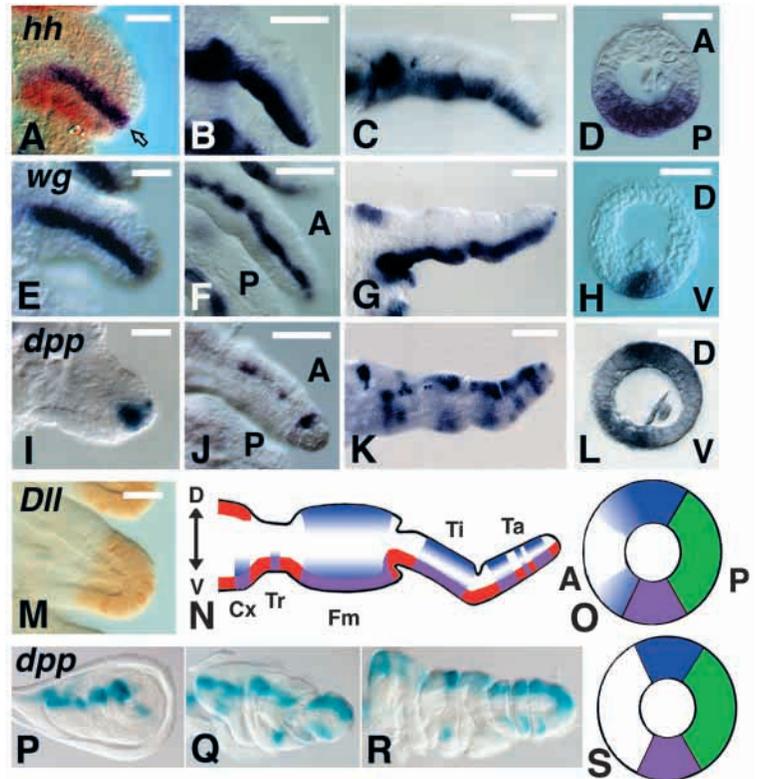
In later stages than stage 11, *dpp* expression patterns in the prothoracic /mesothoracic (T1/T2 leg buds) became different from those in the metathoracic leg bud (T3 leg bud) (Fig. 6). In the femur and tibia of the T1/T2 leg buds, dorsal and ventral stripes of *Gbdpp* expression became intense, while the expression remained circumferential in more distal segments. In transverse sections of the T2 tibia, expression of *Gbdpp* is also found in both dorsal and ventral side intensely (Fig. 5L). However, in the T3 leg bud, circumferential expressions of *Gbdpp* remained intense in the middle of the each segment (Fig. 6).

dpp expression in *Drosophila* imaginal discs

Since expression patterns of *Gbdpp* were considerably different from those of *Dmdpp* in the larval leg imaginal disc, we observed expression patterns of *Dmdpp* in the imaginal disc of the pupa by means of whole-mount in situ hybridization. The expression patterns were similar to the patterns observed when stained with an enhancer trapped line of *dpp-lacZ* BS3.0 (Fig. 5P-R) (Blackman et al, 1991). These results indicate that *Dmdpp* is expressed in a strip running through the dorsal side of the A/P boundary intensely and ventral side weakly in the disc in both larva (Fig. 5P,Q) and pupa (Fig. 5R). However, no circumferential ring of *dpp* expression was detected in these stages. Thus, these results suggest that expression patterns of *dpp* are diverse between the cricket and fly.
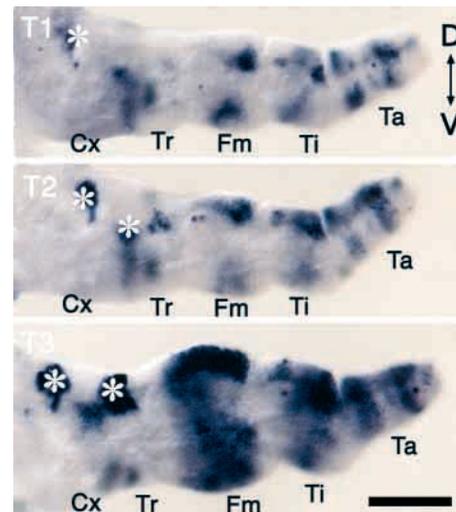

Fig. 6. Expression patterns of *Gbdpp* in *Gryllus* thoracic leg buds at stage 11. The dorsal side is towards the top. In the prothoracic (T1) and mesothoracic (T2) leg buds, *Gbdpp* is expressed as five and seven spots in dorsal and ventral sides, respectively. Location of the spots may correspond to each leg segment of the tarsus (Ta), tibia (Ti), femur (Fm), trochanter (Tr) and coxa (Cx). In the metathoracic (T3) leg bud, *Gbdpp* is expressed as five circumferential bands (three in presumptive Ta, one in each of Ti and Fm) and two ventral spots (one in each of Tr and Cx). Asterisks indicates a nonspecific staining. Scale bars, 100 μm.

Fig. 7. Schematic illustrations of *dpp* expression patterns in the cricket (*Gryllus*), grasshopper (*Schistocerca*) and fly (*Drosophila*) embryos. Expression patterns of *dpp* in the cricket (left), grasshopper (Jockusch et al., 2000) (middle), and fly (right) embryos are arranged from A to F in order of the developmental process. (A) and (B) embryos at leg allocation stages. The anterior side is towards the top. The ventral side is upwards in the *Gryllus* and *Schistocerca* embryos. The lateral side is upwards in the fly embryo. Expression patterns of *wg* are shown in red for each embryo. The boxed region of each embryo is enlarged in the left side in A. Expression patterns of *dpp* in the ventral and dorsal sides of three thoracic segments are shown by blue and light blue, respectively. A shows embryos just before the limb allocation stage. The dorsal longitudinal stripe of *dpp* expression (blue) and ventral stripes of *wg* (red) are commonly observed in the *Gryllus*, *Schistocerca* and *Drosophila* embryos. By contrast, only in the *Schistocerca* embryo, two stripes of *Sadpp* expression are observed in each hemisegment, paralleling the D/V axis. In the Drosophila embryo at stage 11, *Dll* (green) is expressed in each leg primordium. B shows embryos just after the leg allocation stage. Dorsal stripes of *dpp* expression (light blue) are commonly present in both *Gryllus* and *Schistocerca* leg buds. However, in the *Schistocerca* embryos, the ventral *Sadpp* stripe (blue) is observed so as to overlap with the ventral stripes of the *wg* expression (red). In the case of the *Drosophila* embryo, spots of *Dmdpp* expression appear in the dorsal edges of the cluster of cells expressing *Dll*. The dorsal stripe on the disc appears in later stages. (C-F) Leg buds and leg imaginal disc (the dorsal side is towards the top). C and D show leg buds and an imaginal disc, prior to and immediately after the onset of first morphological segmentation. *Gbdpp* is expressed in the dorsal side of the leg bud as a stripe in cricket and as a spot in the *Schistocerca* leg bud. In the Drosophila imaginal disc, *Dmdpp* is expressed as a dorsal stripe in the second instar, then at high levels in the dorsal stripe and much lower levels in the ventral one in the third instar. E shows elongated limb buds of the *Gryllus* and *Schistocerca* embryos. Dorsal spots of *dpp* expression along the P/D axis change to nearly circumferential rings in both *Gryllus* and *Schistocerca* limb buds. The change in the *dpp* expression pattern appears to be correlated with the segmentation of the leg bud. In the *Drosophila* disc, although segmentation progresses during the third instar, but the *Dmdpp* expression pattern does not change to intersegmental rings. F shows metathoracic leg buds after establishment of the leg segmentation. In the metathoracic femur and tibia, the circumferential rings persist until stage 12, and become more intense and wider. However, the circumferential rings of *Gbdpp* expression split into dorsal and ventral stripes in the prothoracic and mesothoracic leg segments. *Dmdpp* expression patterns in the fly disc remained unchanged from the third instar to the pupa. *Sadpp* expression pattern has not been reported in the *Schistocerca* leg buds at the corresponding stage.
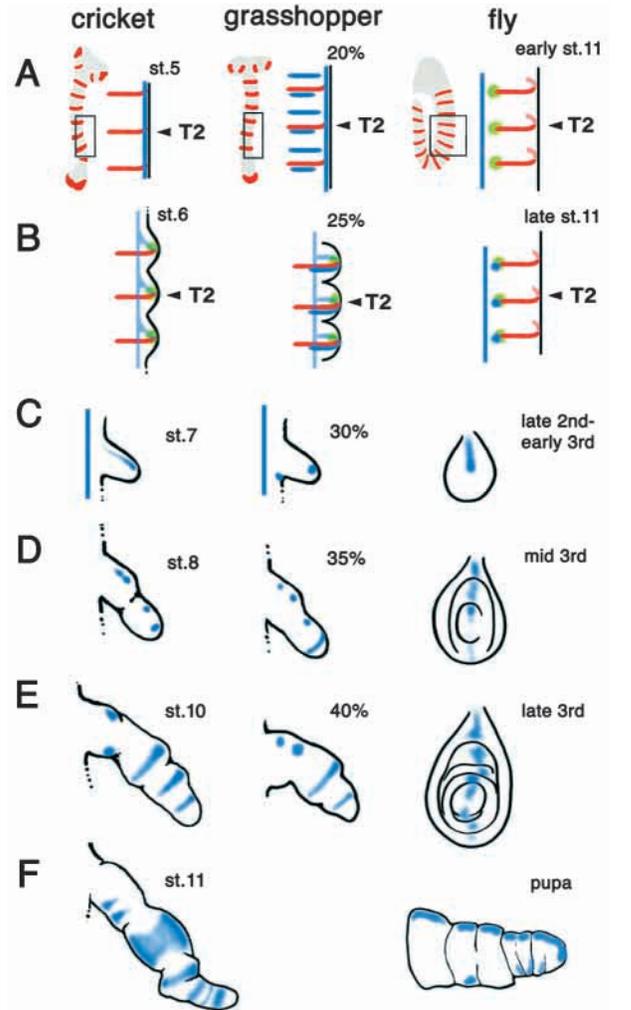
DISCUSSION

In order to examine whether the molecular mechanism of leg development is conserved in the insect, expression patterns of the three signaling molecules of *hh*, *wg* and *dpp* were compared as a first step. The expression profiles of the three genes are found to be essentially conserved in the hemimetabolous short-germ Orthopteran *Gryllus* and *Schistocerca* (Jockusch et al., 2000), holometabolous long-germ Dipteran *Drosophila* and Coleopteran *Tribolium* (*Tcwg*, Nagy and Carroll, 1994; *Tcdpp*, Sanchez-Salazar et al., 1996), suggesting the developmental mechanism is common in principle in the insect. However, we found that expression pattern of *dpp* during leg development was divergent, although expression patterns of *hh* and *wg* were essentially conserved. For comparison, the expression patterns of *dpp* in *Gryllus*, *Drosophila* and *Schistocerca* are schematically illustrated in Fig. 7, in which the processes of insect leg development are divided into two phases: Phase 1 – Allocation of the leg primordium; Phase 2 – leg patterning. In

the following, the divergence of *dpp* expression patterns and its correlation with diversity of leg morphology are discussed for each phase.

Leg allocation in Phase 1

In the allocation phase of *Drosophila* 5h embryos, *Dmwg* and *Dmhh* are expressed in a stripe along the anteroposterior (A/P) compartment boundary (Fig. 7A) and in the posterior region of each segment, respectively (French and Daniels, 1994; Campbell and Tomlinson, 1995 as reviews). However, *Dmdpp* is expressed throughout the dorsal region (Fig. 7A) and then in the dorsal side of the *wg* stripe (Fig. 7B) (St Johnston and Gelbart, 1987). Later, the expression changes to give two thin stripes running anteroposteriorly along the length of the embryo (Fig. 1A) (St Johnston and Gelbart, 1987). Recently, Goto and Hayashi (1997) demonstrated that WG, but not DPP, was responsible for initial specification of the limb primordia with a distal identity and for induction of *Dll*. They proposed a model for the allocation of the limb primordium (the G-H

model; Goto and Hayashi, 1997) (Fig. 7A,B). A stripe of WG induces the limb primordium expressing *Dll*. Repression of *Dll* by DPP from the dorsal side and by Spits (*Drosophila* EGF) from the ventral side limits the limb formation only in the lateral position. Then, DPP specifies proximal cell identity in the primordium in a concentration-dependent manner.

In *Gryllus* and *Schistocerca* embryos, expression of *wg* was detected in a stripe along the A/P compartment boundary of the body segment (Fig. 7A). In *Gryllus* embryos, expression of *dpp* was first detected along the periphery of the germ band (Fig. 7A). Similar expression patterns were observed also in *Tribolium* (Sanchez-Salazar et al., 1996). Although the cricket and grasshopper belong to the same Orthoptera, the expression patterns of *Sadpp* (Jockusch et al., 2000) are more complicated than those of *Gbdpp* (Fig. 7). In *Schistocerca* embryos at early stages, *Sadpp* is expressed in two partial stripes in each hemisegment, intrasegmentally and intersegmentally, paralleling the D/V axis (Jockusch et al., 2000) (Fig. 7A). The intrasegmental stripes extend along both dorsal and ventral sides of the presumptive leg field (Fig. 7B). Early expression patterns of *Gbdpp* resemble those of *Dmdpp* or *Tcdpp* more closely than those of *Sadpp* (Fig. 7A,B). Thus, the *wg* expression pattern appears conserved in the allocation phase, while early expression patterns of *dpp* seem divergent even in the Orthoptera. Thus, more data are necessary to judge whether the G-H model is also applicable as a model for initiation of limb formation in other insects.

Patterning along the proximodistal axis in Phase 2

In the Phase 2, in the *Drosophila* leg imaginal disc, *Dmhh* is expressed in the posterior compartment of the disc, determining the A/P pattern, and induces *Dmdpp* and *Dmwg* expression in the dorsal and ventral side of the A/P boundary, respectively (Figs 1B and 7C) (Campbell et al., 1993; Basler and Struhl, 1994; Diaz-Benjumea et al., 1994). They act cooperatively in a concentration-dependent manner to organize the P/D axis and induce expression of *Dll* at the center of the disc (Lecuit and Cohen, 1997). In *Gryllus* and *Schistocerca* limb buds, since *hh* and *wg* are expressed in the posterior and the ventral side of the A/P boundary, respectively, their functions during limb development should be conserved among the fly, cricket, beetle and grasshopper.

However, expression patterns of *Gbdpp* are considerably different from those of *Dmdpp*: *Gbdpp* expression is limited to a dorsal stripe transiently around the time of limb bud emerging at stage 6-7 (Figs 3B and 7B), and at this time, expression of *Dll* was found in the distal tip of the limb bud (Fig. 5M). This transient expression pattern was also reported in *Schistocerca* embryos (Fig. 7B). 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, indicating that Dpp is required for the initiation but not maintenance of *Dll* transcription (Lecuit and Cohen, 1997). Thus, it is reasonable to consider that transient *dpp* expression is enough to induce expression of *Dll*, which is required for the P/D leg pattern formation.

In the *Drosophila* leg imaginal disc, *Dmdpp* expression is restricted as a stripe in the dorsal side of the anterior compartment (Fig. 1B) (Jiang and Struhl, 1996; Penton and Hoffmann, 1996; Brook and Cohen, 1996). HH was demonstrated to induce expression of *Dmdpp* in the dorsal side

along the A/P boundary (Jiang and Struhl, 1996; Penton and Hoffmann, 1996; Brook and Cohen, 1996), and is repressed in the posterior leg compartment by the EN protein (Sanicola et al, 1995). In the *Gryllus* leg buds, however, *Gbdpp* is expressed as four spots in the dorsal side of the A/P boundary at stage 9 (Figs 5J and 7D), then expression domains transform into a nearly circumferential ring at stages 10-11 (Figs 5K, and 7E,F), which is located roughly in the middle of the primary leg segments, as observed in *Schistocerca*. Since *Gb*EN (Niwa et al., 1997) and *Gbhh* are always expressed throughout the posterior compartment, the relation between the expression domain of *Dmdpp* with that of *Dmhh/Dmen* does not hold true in the *Gryllus* leg bud, indicating divergent roles of *dpp* in leg patterning. Jockusch et al. (2000) proposed an idea that *Sadpp* may play roles in establishment of boundaries between the leg segments, because the appearance of segmentally reiterated rings of *Sadpp* precedes morphological segmentation of leg buds (Fig. 7E). This idea may be applicable to *Gryllus* leg buds.

For the patterning along the proximodistal (P/D) axis in the *Drosophila* limb disc, two models have been proposed: the gradient model (Lecuit and Cohen, 1997; Abu-Shaar and Mann, 1998; Wu and Cohen, 1999) and the intercalation model (Goto and Hayashi, 1999). In the gradient model, it is proposed that since WG and DPP are secreted from the respective ventral and dorsal halves of the cells at the A/P compartment boundary, forming a gradient along the P/D axis, differential response to this gradient leads to circular patterns corresponding to the leg segments. However, in the intercalation model, they proposed that after formation of extreme proximal and distal structures by DPP and WG, the intermediate leg segments are intercalated by a signal from the proximal domain (Goto and Hayashi, 1999). In the case of *Gryllus* limb buds, the intercalation model appears favorable, because the discrete expression of *Gbdpp* can not maintain the DPP gradient in the limb bud necessary for circular patterns corresponding to the leg segments. Furthermore, intercalation of the leg segments occurs during development of *Gryllus* leg buds (Fig. 1E): the most distal part is formed at first, then limb segmentation occurs at the coxopodite/telopodite boundary, followed by the femur/tibia, trochanter/femur and tibia/tarsus boundaries, judged from the expression patterns of DLL (Niwa et al., 1997), *Gryllus homothorax*, *dachshund* and *aristaless* (data not shown). These results are consistent with those observed in the *Schistocerca* leg buds, using an Annulin antibody (Singer et al., 1992) for detection of the limb segment boundary. The intercalation model may be supported also by the fact that regeneration of amputated cricket legs occurs through intercalation of the missing segments (data not shown) as observed in the cockroach leg (French et al., 1976).

Patterning along the dorsoventral axis in Phase 2

For establishment of the dorsoventral pattern of the *Drosophila* leg disc, it has been suggested that the two signals of WG and DPP act antagonistically to repress each other's expression and to specify dorsal and ventral expression patterns (Jiang and Struhl, 1996; Penton and Hoffmann, 1996; Brook and Cohen, 1996). However, in later stages, in early third instar to early pupal leg discs of *Drosophila*, *Dmdpp* is expressed in both dorsal and ventral sides (Figs 5P-Q, and 7E,F), as reported by Masucci et al. (1990) and Jockusch et al. (2000). This faint ventral expression in the *Dmwg* expression domain may

correspond to the ventral expression of *Gbdpp* in the region where *Gbwg* was expressed intensely (Figs 5H,L and 7E,F). These observations indicate that expression of the two genes may be regulated independently in later stages.

It is interesting to note that the *Gbdpp* expression pattern changes from rings to the intense dorsal and ventral stripes (Fig. 6), when each leg segment begins to change from a circular shape to an elliptical shape extended dorsoventrally at stage 11. Thus, the dorsal and ventral expressions of *dpp* are likely to correlate with the dorsoventral extension of the leg bud. Furthermore, we found that the *Gbdpp* expression patterns were different between the metathoracic (T3) leg bud and pro/mesothoracic (T1/T2) leg buds in stage 11-12 (Fig. 6): the circumferential band of *Gbdpp* expression in the femur and tibia of the T3 leg segment became more intense and wider than that in the femur and tibia of the T1/T2 leg segments. This may be related to the fact that the *Gryllus* adult T3 leg, which has large muscles in the dorsal and ventral side of the femur and tibia for jumping, is several times larger than the T1 and T2 legs. Since the appearance of these segmentally reiterated rings of *Gbdpp* precedes elongation of leg segments (Fig. 7E,F), *Gbdpp* may be involved in outgrowth of the leg segment. We speculated that the similar difference in expression patterns of *Sadpp* among the three legs should be observed in the *Schistocerca* embryo. However, since expression patterns of *Sadpp* in the corresponding stages were not reported so far, we cannot verify our speculation. In *Drosophila*, in which there is little difference in morphology among the *Drosophila* thoracic legs, no difference is reported in expression patterns of *Dmdpp* among the three leg imaginal discs. This is consistent with our speculation. Thus, the expression patterns of *dpp* are likely to correlate with leg morphology, implying that temporal and spatial regulations of the *dpp* gene expression are closely correlated with morphological diversity.

In conclusion, although leg developmental pathways are different among insect species, the principal mechanism of leg development is likely to be conserved, judged from expression patterns of *hh, wg* and *dpp*. Divergence in *dpp* expression patterns during leg development is likely to correlate with morphological diversity of the leg.

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.

Akam, M. (1994). Insect development. Is pairing the rule? *Nature* **367**, 410-411.

Akam, M. (1998). The Yin and Yang of Evo/Devo. *Cell* **92**, 153-155.

Akam, M. and Dawes, R. (1992). More than one way to slice an egg. *Curr. Biol.* **2**, 395-398.

Arora, K., Levine, M. S. and O'Connor M. B. (1994). The *screw* gene encodes a ubiquitously expressed member of the TGF-beta family required for specification of dorsal cell fates in the *Drosophila* embryo. *Genes Dev.* **8**, 2588-2601.

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

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-β family in *Drosophila*. *Development* **111**, 657-666.

Brook, W. J. and Cohen, S. M. (1996). Antagonistic interactions between wingless and decapentaplegic responsible for dorsal-ventral pattern in the *Drosophila* leg. *Science* **273**, 1373-1377.

Campbell, G. and Tomlinson, A. (1995). Initiation of the proximodistal axis in insect legs. *Development* **121**, 619-628.

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.

Cohen, S. M., Bronner, G., Kuttner, F., Jurgens, G. and Jackle, H. (1989). *Distal-less* encodes a homoeodomain protein required for limb development in *Drosophila*. *Nature* **338**, 432-434.

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.

Dietrich, S., Schubert, F. R. and Lumsden, A. (1997). Control of dorsoventral pattern in the chick paraxial mesoderm. *Development* **124**, 3895-3908.

Doctor, J. S., Jackson, P. D., Rashka, K. E., Visalli, M. and Hoffmann, F. M. (1992). Sequence, biochemical characterization, and developmental expression of a new member of the TGF-β superfamily in *Drosophila melanogaster*. *Dev. Biol.* **151**, 491-505.

Engel, J. E. and Hoy, R. R. (1999). Experience-dependent modification of ultrasound auditory processing in a cricket escape response. *J. Exp. Biol.* **202**, 2797-2806.

French, V., Bryant, P. J. and Bryant, S. V. (1976). Pattern regulation in epimorphic fields. *Science* **193**, 969-981.

French, V. and Daniels, G. (1994). Pattern formation. The beginning and the end of insect limbs. *Curr. Biol.* **4**, 34-37.

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

Goto, S. and Hayashi, S. (1999). Proximal to distal cell communication in the *Drosophila* leg provides a basis for an intercalary mechanism of limb patterning. *Development* **126**, 3407-3413.

Hogan, B. L. (1999). Morphogenesis. *Cell* **96**, 225-233.

Jiang, J. and Struhl, G. (1996). Complementary and mutually exclusive activities of decapentaplegic and wingless organize axial patterning during *Drosophila* leg development. *Cell* **86**, 401-409.

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.

Lawrence, P. A. and Struhl, G. (1996). Morphogens, compartments, and pattern: lessons from *Drosophila*? *Cell* **85**, 951-961.

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

Masucci, J. D., Miltenberger, R. J. and Hoffman, F. M. (1990). Pattern specific expression of the *decapentaplegic* gene in the imaginal discs is regulated by 3′ cis regulatory elements. *Genes Dev.* **4**, 2011-2023.

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-β family members in the grasshopper *Schistocerca americana*. *J. Mol. Evol.* **41**, 155-160.

Niwa, N., Saitoh, M., Ohuchi, H., Yoshioka, H. and Noji, S. (1997). Correlation between *Distal-less* expression patterns and structures of appendages in development of the two-spotted cricket, *Gryllus bimaculatus*. *Zool. Sci.* **14**, 115-125.

Padgett, R.W., St Johnston, R. D. and Gelbart, W. M. (1987). A transcript from a *Drosophila* pattern gene predicts a protein homologous to the transforming growth factor-β family. *Nature* **325**, 81-84.

Patel, N. H., Martin-Blanco, E., Coleman, K. G., Poole, S. J., Ellis, M. C., Kornberg, T. B. and Goodman, C. S. (1989). Expression of *engrailed* proteins in arthropods, annelids, and chordates. *Cell* **58**, 955-968.

Patel, N. H. (1993). Evolution of insect pattern formation: a molecular analysis of short germband segmentation. In *Evolutionary Conservation of Developmental Mechanisms* (ed. A. Sprading), pp. 85-110. New York: Wiley-Liss.

Patel, N. H. (1994). Evolution of insect patterning. *Proc. Natl. Acad. Sci. USA* **91**, 7385-7386.

Paydar, S., Doan, C. A. and Jacobs, G. A. (1999). Neural mapping of direction and frequency in the cricket cercal sensory system. *J. Neurosci.* **19**, 1771-1781.

Penton, A. and Hoffmann, F. M. (1996). Decapentaplegic restricts the domain of wingless during *Drosophila* limb patterning. *Nature* **382**, 162-164.

Rijsewijk, F., Schuermann, M., Wagenaar, E., Parren, P., Weigel, D. and Nusse, R. (1987). The Drosophila homolog of the mouse mammary oncogene *int-1* is identical to the segment polarity gene *wingless*. *Cell* **50**, 649-657.

Sanchez-Salazar, J., Pletcher, M. T., Bennett, R. L., Brown, S. J., Dandamudi, T. J., Denell, R. E. and Doctor, J. S. (1996). The *Tribolium decapentaplegic* gene is similar in sequence, structure, and expression to the *Drosophila dpp* gene. *Dev. Genes Evol.* **206**, 237-246.

Sanicola, M., Sekelsky, J., Elson, S. and Gelbart, W. (1995). Drawing a stripe in *Drosophila* imaginal disks: negative regulation of *decapentaplegic* and *patched* expression by *engrailed*. *Genetics* **139**, 745-756.

Sasaki, H. and Hogan, B. L. (1993). Differential expression of multiple fork head related genes during gastrulation and axial pattern formation in the mouse embryo. *Development* **118**, 47-59.

Singer, M. A., Hortsch, M., Goodman, C. S. and Bentley, D. (1992). Annulin, a protein expressed at limb segment boundaries in the grasshopper embryo, is homologous to protein cross-linking transglutaminases. *Dev Biol.* **154**, 143-159.

St Johnston, R. D. and Gelbart, W. M. (1987). *Decapentaplegic* transcripts are localized along the dorsal-ventral axis of the *Drosophila* embryo. *EMBO J.* **6**, 2785-2791.

Tashiro, S., Michiue, T., Higashijima, S., Zenno, S., Ishimaru, S., Takahashi, F., Orihara, M., Kojima, T. and Saigo, K. (1993). Structure and expression of *hedgehog*, a *Drosophila* segment-polarity gene required for cell-cell communication. *Gene* **124**, 183-189.

Wenzel, B. and Hedwig, B. (1999). Neurochemical control of cricket stridulation revealed by pharmacological microinjections into the brain. *J. Exp. Biol.* **202**, 2203-2216.

Wharton, K. A., Thomsen, G. H. and Gelbart, W. M. (1991). *Drosophila 60A* gene, another transforming growth factor beta family member, is closely related to human bone morphogenetic proteins. *Proc. Natl. Acad. Sci. USA* **88**, 9214-9218.

Wilkinson, D. G. (1992). A practical approach. In *In Situ Hybridization* (ed. D. G. Wilkinson), pp. 75-83. Oxford: IRL Press.

Wu, J. and Cohen, S. M. (1999). Proximodistal axis formation in the *Drosophila* leg: subdivision into proximal and distal domains by Homothorax and Distal-less. *Development* **126**, 109-117.