Dev Genes Evol (2001) 211:232–243
DOI 10.1007/s004270100150

ORIGINAL ARTICLE

Beate Mittmann · Gerhard Scholtz

Distal-less expression in embryos of *Limulus polyphemus* (Chelicerata, Xiphosura) and *Lepisma saccharina* (Insecta, Zygentoma) suggests a role in the development of mechanoreceptors, chemoreceptors, and the CNS

Received: 30 October 2000 / Accepted: 5 February 2001 / Published online: 24 March 2001

Abstract The homeobox gene *Distal-less* (*Dll*) is well known for its participation in the development of arthropod limbs and their derivatives. *Dll* activity has been described for all groups of arthropods, but also for molluscs, echinoderms and vertebrates. Generally, *Dll* participates in the establishment of the proximo-distal-axis and differentiation along this axis. During our investigation of the expression pattern in the silverfish *Lepisma saccharina* and the horseshoe crab *Limulus polyphemus*, we found several expressions in late stages which cannot be explained with the "normal" limb-specific function. The antenna, cerci and terminal filament of the silverfish show a striped expression; single cells on the labrum, mandibles, maxillary palps and anal valves are also strongly stained by the *Dll* antibody. In addition to cell groups in the developing ganglia of the CNS, in the coxal endites and several nerve cells in femur and the trochanter of the prosomal limbs, the whole prosomal shield of *Limulus polyphemus* is surrounded by *Dll*-positive cell clusters. Furthermore, the lateral processes of the opisthosoma and the edges of the opisthosomal appendages are *Dll* positive. To get an indication of the cell fate of these regions, we examined hatched larvae and juvenile stages of both species with the SEM. We found a striking correlation of these *Dll*-positive areas and different sense organs, especially mechanoreceptors. Since many sense organs in arthropods are situated on the limbs, interpretation of the *Dll* expression in limbs is problematical. This has critical implications for comparative analysis of *Dll* expression patterns between arthropods and for the claim of homology between limb-like structures. Furthermore, we discuss the possibility of convergent appendage evolution in various bilaterian groups based on the improvement of spatial sensory resolution.

Keywords Limb development · Neurogenesis · Sensory organs · Evolution · Central nervous system

Edited by D. Tautz

B. Mittmann (✉) · G. Scholtz
Humboldt-Universität zu Berlin, Institut für Biologie, Vergleichende Zoologie, Philippstrasse 13, 10115 Berlin, Germany
e-mail: beate=mittmann@rz.hu-berlin.de
Fax: +49-30-20936002

Introduction

The homeobox gene *Distal-less* (*Dll*) has been found to be expressed in representatives of all higher arthropod groups: in onychophorans, chelicerates, crustaceans, myriapods and insects (Panganiban et al. 1994, 1995, 1997; Popadic et al. 1996, 1998; Williams and Nagy 1996; Grenier et al. 1997; Niwa et al. 1997; Scholtz et al. 1998; Williams 1998; Thomas and Telford 1999; Mittmann 2000). *Dll* is a transcription factor that is activated several times during development and participates in the differentiation of various organ systems. Its best known and best investigated function lies in the development of the extremities and their derivatives, where it acts in two ways: first as a kind of genetic switch, which enables particular cells to become precursor cells of extremities (Cohen and Jürgens 1989a, b), and second in the establishment of the proximo-distal axis (pd-axis) and differentiation along this axis (Sunkel and Whittle 1987; Cohen et al. 1989) by specifying the distal cell fate (Cohen and Jürgens 1989a,b; Gorfinkiel et al. 1997; Campbell and Tomlinson 1998; Wu and Cohen 1999; Dong et al. 2000). The former idea that *Dll* is expressed in a gradient along the pd-axis with the main expression at the tip (Cohen and Jürgens 1989a,b; Cohen et al. 1989) does not seem to be supported by more recent investigations (Panganiban 2000). Apart from its function during the outgrowth of extremities, there is some evidence that *Dll* also participates in the development of sensory organs, such as the Keilin's organs and antennal sense organs, together with that of the central nervous system (Cohen et al. 1989; Vachon et al. 1992; Kaphingst and Kunes 1994; Panganiban et al. 1997;

Mittmann 2000; Panganiban 2000), but this involvement has not yet been well examined.

The largest domain of the early expression of *Dll* shows a similar pattern in many arthropod species examined and is apparently related to the development of limb buds and of the pd-axis during limb growth. However, there are *Dll* expression patterns in limbs during later stages of several arthropod species which are more difficult to explain in terms of axis formation. In the prolegs of the butterfly *Precis coenia*, the expression of *Dll* after the establishment of the pd-axis is restricted to a proximal ring; the more distal expression is lacking or highly reduced in level (Panganiban et al. 1994). The imaginal discs of *Drosophila melanogaster* show a late expression of *Dll* in the proximal ring that corresponds to the trochanter and a part of the femur (Gonzáles-Crespo and Morata 1996). In the thoracic legs and the maxillary palps of the cricket *Gryllus bimaculatus*, *Dll* is expressed in a "striped" pattern: the distal region, which expresses *Dll*, is followed by a region without expression and a more proximal area, where *Dll* again is active (Niwa et al. 1997). In crustaceans with foliaceous limbs, *Dll* is expressed in the anlagen of the lobe-like endites (Panganiban et al. 1995; Williams 1998). A similar pattern has been found in the endites of crustacean and insect maxillae (Niwa et al. 1997; Scholtz et al. 1998) and in the endites of arachnid pedipalps (Thomas and Telford 1999). The proximal domain of all given examples could be caused by the *Dll* product being required for the expression of other genes involved in the pd-patterning of appendages. It has furthermore been suggested that *Dll* plays a general role in the outgrowth of structures in animals (Panganiban et al. 1997); this could explain *Dll* expression in arthropod endites. In the case of the *Drosophila* imaginal discs, the *Dll* expression seems to be necessary to avoid a mixing of proximal and medial cells (Wu and Cohen 1999).

The present paper deals with aspects of the spatial and temporal expression patterns of *Dll* in embryos of the horseshoe crab *Limulus polyphemus* and the apterygote insect *Lepisma saccharina* using a polyclonal antibody against the *Dll* protein (Panganiban et al. 1995). We focus our examinations on the later embryogenesis. To get an indication of the fate of the areas expressing *Dll* in advanced stages, we also investigated freshly hatched larvae and later larval stages of both species by combining antibody staining and scanning electron microscopy. We show that there is a general correlation of *Dll* expression and the development of various sense organs and the CNS in arthropods. This suggests a role for *Dll* in the formation of these structures in arthropods. Since in arthropods many sense organs are situated on the limbs and their derivatives, the interpretation of the *Dll* expression patterns in limbs is problematical. Is a specific pattern of *Dll* expression related to limb growth and specification or, rather, does it reflect the differentiation of sensory structures? This has critical implications for comparative analyses of *Dll* expression patterns between arthropods and, in particular, for the claim of homology between limb-like structures. We discuss the possibility of convergent appendage evolution in various bilaterian groups based on the improvement of spatial sensory resolution.

Materials and methods

Lepisma saccharina is one of our breeding animals in Berlin (detailed description on request), whereas the eggs of *Limulus polyphemus* were collected in Woods Hole (Massachusetts). In the eggs of both species, the chorion and yolk were removed with insect pins and tweezers. Immunostaining with some modifications followed the description of Panganiban (personal communication). The embryos were transferred to the fixative [*Lepisma saccharina*: PEM-FA: 01 M PIPES (pH 7.4), 2.0 mM EGTA, 0.1 mM $MgSO_4$, 3.7% formaldehyde for 30–60 min; *Limulus*: PBS-FA: PBS, 3.7% formaldehyde for 4–12 h). After fixation, the embryos were washed several times in PBS and PBT (PBS, 0.2% BSA, 0.1% Triton X-100) and kept for at least 30 min in PBT. Very late stages were sonicated 3 times for 10 s before incubating in PBT and polyclonal anti*Dll* (dilution 200:1) for at least overnight at 4°C. After incubation, they were washed several times for 10 min and at least 4 times for 30 min in PBT; then they were incubated overnight at 4°C in PBT and peroxidase-conjugated goat antirabbit IgG (Jackson Immunoresearch, 800:1). After this incubation, the embryos were washed for several hours in PBT and transferred to a solution of 1 mg/ml DAB (diaminobenzidine) in PBT (dilution 2:1) for 10 min (*Lepisma*) or 20 min (*Limulus*). H_2O_2 was added to a dilution of 100:1 and the reaction was allowed to proceed for about 10–30 min. The stained embryos were transferred to PBS and counterstained with fluorescent dye (0.1% to 1% bisbenzimid H33258) for 10 min (*Lepisma*) or about 2 h (*Limulus*). Further analyses were performed with brightfield, differential interference contrast (Nomarski optics) and fluorescence microscopy (Zeiss Axiophot).

For scanning electron microscopy, embryos and larvae were fixed in Bouin (Romeis 1989) for at least 12 h, washed several times in EtOH (70%) and dehydrated through an alcohol series. Afterwards, they were critical point dried in CO_2 (CPD BAT-TEC 030), mounted and sputter coated (SCD BAL-TEC 005) with a layer of gold. All images were produced with a Leica scanning electron microscope.

Results

General pattern

In early stages, the extremities and their derivatives of *Lepisma saccharina* and *Limulus polyphemus* show the "typical" *Dll* expression, which is well known from other arthropods. In *Lepisma saccharina*, we found a premorphogenetic expression, followed by an expression in the distal domain of the outgrowing buds, in all segments bearing appendages except that of the mandible (Fig. 1A; Scholtz et al. 1998; Mittmann 2000). In the earliest *Limulus* embryos examined, the prosomal appendages had already started to bud and expressed *Dll* at their tips (Fig. 3A, B). In both species the labrum is also *Dll* positive. In later stages, this "normal" expression of the appendages mentioned is replaced by a different pattern.

Lepisma saccharina

Antibody staining

The first segment of the antenna is nearly all *Dll* negative with the exception of a few cells, while the second

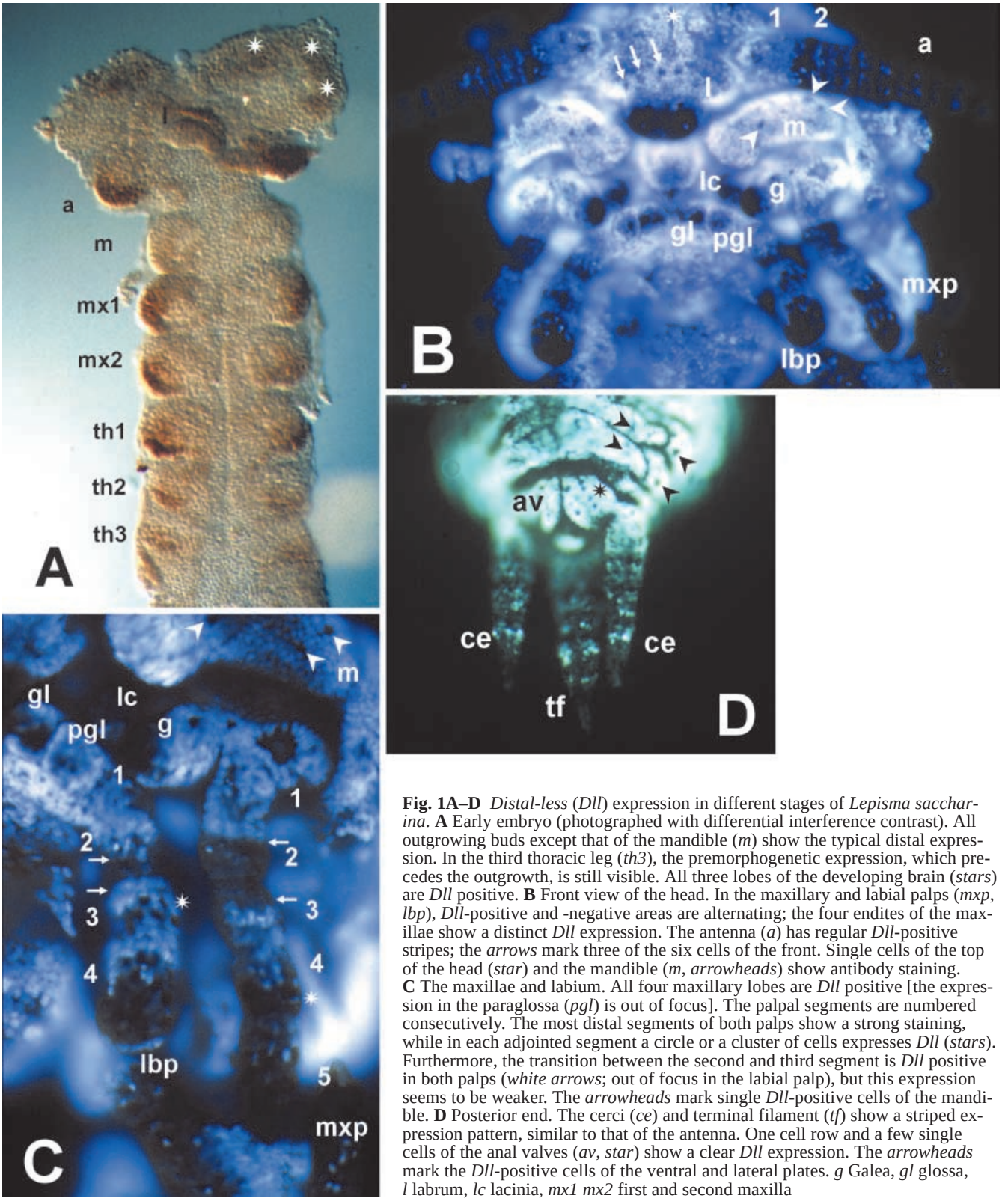


Fig. 1A–D *Distal-less* (*Dll*) expression in different stages of *Lepisma saccharina*. **A** Early embryo (photographed with differential interference contrast). All outgrowing buds except that of the mandible (*m*) show the typical distal expression. In the third thoracic leg (*th3*), the premorphogenetic expression, which precedes the outgrowth, is still visible. All three lobes of the developing brain (*stars*) are *Dll* positive. **B** Front view of the head. In the maxillary and labial palps (*mxp*, *lbp*), *Dll*-positive and -negative areas are alternating; the four endites of the maxillae show a distinct *Dll* expression. The antenna (*a*) has regular *Dll*-positive stripes; the *arrows* mark three of the six cells of the front. Single cells of the top of the head (*star*) and the mandible (*m*, *arrowheads*) show antibody staining. **C** The maxillae and labium. All four maxillary lobes are *Dll* positive [the expression in the paraglossa (*pgl*) is out of focus]. The palpal segments are numbered consecutively. The most distal segments of both palps show a strong staining, while in each adjointed segment a circle or a cluster of cells expresses *Dll* (*stars*). Furthermore, the transition between the second and third segment is *Dll* positive in both palps (*white arrows*; out of focus in the labial palp), but this expression seems to be weaker. The *arrowheads* mark single *Dll*-positive cells of the mandible. **D** Posterior end. The cerci (*ce*) and terminal filament (*tf*) show a striped expression pattern, similar to that of the antenna. One cell row and a few single cells of the anal valves (*av*, *star*) show a clear *Dll* expression. The *arrowheads* mark the *Dll*-positive cells of the ventral and lateral plates. *g* Galea, *gl* glossa, *l* labrum, *lc* lacinia, *mx1 mx2* first and second maxilla

segment shows a strong staining. The rest of the antenna expresses *Dll* in regular stripes, but over and above that there are single cells with a very strong staining in each annulus (Fig. 1B). The distal part of the labrum is *Dll* positive, and near the transition to the *Dll*-negative proximal area, several cells seem to show a stronger expression. Furthermore, six isolated cells at the front of the head and two cells near the top of the head show a dis-

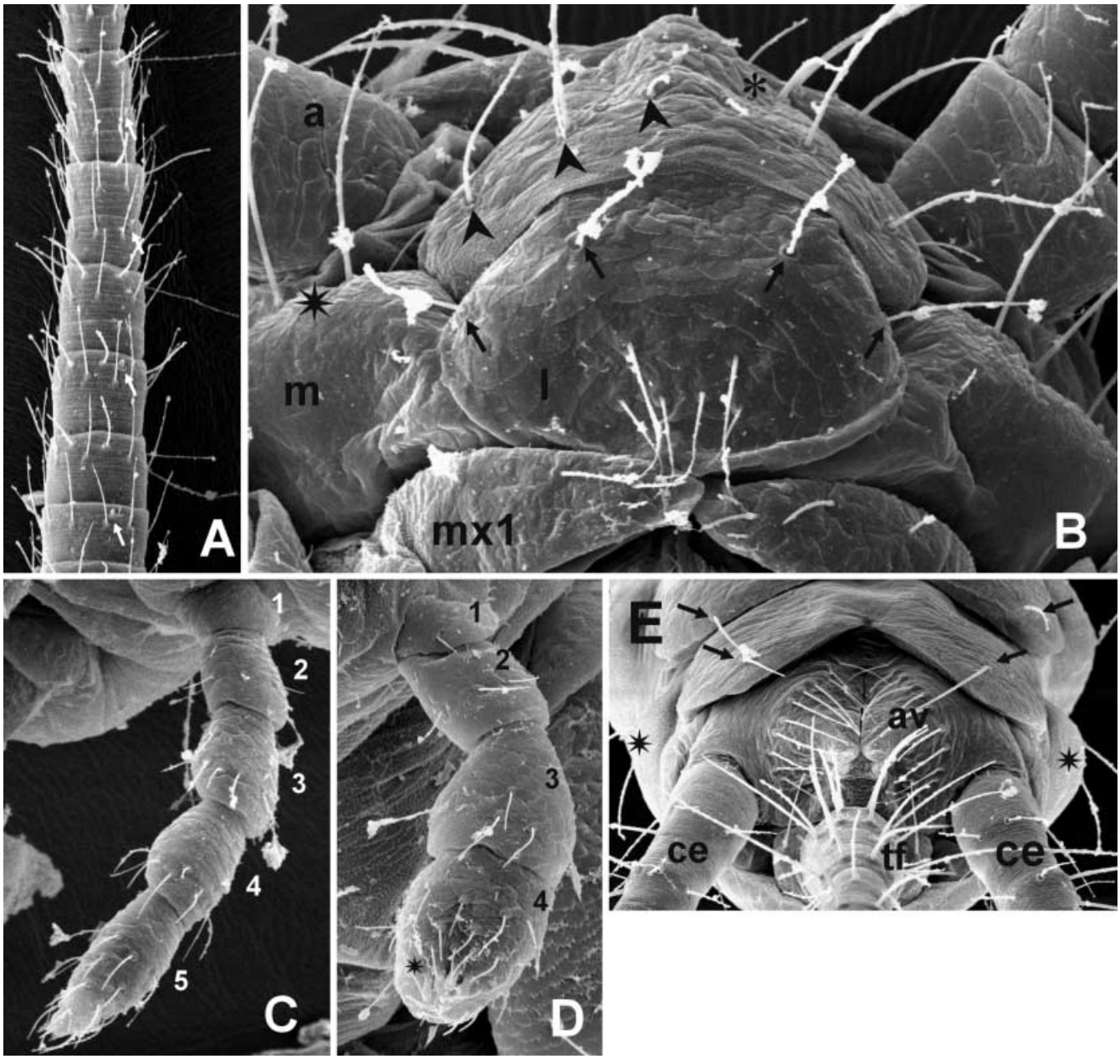


Fig. 2A–E SEM of postembryonic stages of *Lepisma saccharina*. **A** Middle part of the antenna. Each annulus of the antenna bears a circle of setae. The sensilla basiconica (*arrows*) occur in this stage at least in every second segment. **B** Frontal view of the head. The labrum (*l*) shows six setae at the tip and four single setae (*arrows*) in the proximal area. The *arrowheads* mark three of the six setae on the front; just one of the two setae at the top of the head is visible (*asterisk*). Beside the *star*, there are two of the three mandibular setae. **C** The maxillary palp. While the distal area of the fifth segment is covered with setae, there is only a circle of setae in the distal part of the fourth, third and second segments. **D** The labial palp. The distal part of the fourth segment is covered with different sense organs. The biggest ones are the five complex sensilla (*star*). The second and third segment bear one circle of setae; near the ring of setae of the third segment, there is a single seta. **E** The posterior end of the first instar. The cerci (*ce*) and the terminal filament (*tf*) bear circles of setae, while there is one row of setae on each anal valve (*av*). The *arrows* mark the setae of the ventral plates, the *stars* those of the lateral plates. *a* Antenna, *m* mandible, *mx1* first maxilla

tinct gene expression (Fig. 1B). Even though the mandible is *Dll* negative in all earlier stages examined (Scholtz et al. 1998; Mittmann 2000), we found three isolated cells showing *Dll* activity in the very late stages (Fig. 1B, C). The first maxillary palp consists of five segments. We found *Dll* expression in the distal region of the fifth segment, in a circle of cells in the fourth segment, in a few distally situated cells of the third segment, and in an area around the transition between the third and second segment (Fig. 1C). The lacinia and the galea as well as the glossa and the paraglossa of the second maxillae (labium) also express *Dll* (Fig. 1B, C). This is the first evidence for *Dll* expression in the paraglossa of an insect. The labial palp consists of four segments, if the so-called palpiger is regarded as the first segment and not as part of the praementum. Nearly the whole dis-

tal part of the fourth segment is *Dll* positive, followed by a narrow proximal area without *Dll* activity (Fig. 1C). In the third segment, only a few cells express the gene. Similar to the first maxillary palp, there is an expression domain near the transition of the second and third segment. In both palps, this expression seems to be weaker than that of the single cells. The first segment shows no *Dll* expression or just a very reduced one. The cerci and the terminal filament also show regular stripes of *Dll*-expressing cells across the whole length except for the most proximal part (Fig. 1D). Furthermore, one cell row and a few single cells on each anal valve and some single cells on both sides of each abdominal segment show a distinct expression of the gene (Fig. 1D).

In the central nervous system, *Dll* can be found in all three lobes of the developing brain (Fig. 1A).

Scanning electron microscopy

To determine the post-hatching fate of *Dll*-positive domains of late embryogenesis, we used scanning electron microscopy and found a striking correspondence between the position of different receptors and the position of *Dll*-expressing cells. The sense organs on the antenna show a great variety; Adel (1984) described nine different types of receptors. Because their names differ from author to author and some mechanoreceptors change into different types during postembryonic development, in some cases we use common names; otherwise, we follow the naming of Larink (1982, 1983).

In the antenna of *Lepisma saccharina*, the second segment and all annuli except the third and the fifth bear a ring of setae. In addition, there are egg-shaped sense organs in at least every second annulus (Fig. 2A). According to Larink (1982) these are setae, sensilla trichobothria, and sensilla basiconica; the latter are chemoreceptors. In later stages, the first and the third annulus also bear rings of setae. In the hatching stage, we found four setae on the labrum in a proximal position and another cluster of six setae near the tip. Furthermore, the head bears three pairs of setae on the front and two pairs of different-sized setae on the top (Fig. 2B). All these setae increase in number during further development. On the lateral basal part of the mandibles, there are three bristles (Fig. 2B). The distal region of the fifth segment of the first maxillary palp is covered with different sense organs (mechanoreceptors, chemoreceptors, glandular hairs), while there is just one circle of setae in the fourth, third and second segment (Fig. 2C). The investigation of the labial palp provided a similar result. Again the distal part of the last segment is covered with different types of sensilla (Fig. 2D); most of them are sensilla trichodea, i.e. sharp, flexed bristles. In addition, there are several types of sensilla basiconica (Larink 1978). Furthermore, there are five so-called complex sensilla (Larink 1978, 1982, 1983) at the tip (Fig. 2D) which are probably chemoreceptors (Larink 1978). On the third segment we found just a few setae, while there exists a ring of setae on the second segment, but only one bristle on the first segment (Fig. 2D). The number of the palpal sensilla increases during postembryonic development (Larink 1978). The cerci and the terminal filament, except in their most proximal areas, are covered with rings of movable jointed setae in the distal part of each segment that differ noticeably in size (Fig. 2E). The two laminae subanales show a row of setae (Fig. 2E). In each abdominal segment, we found several setae on each side of the ventral and lateral plates (Fig. 2E).

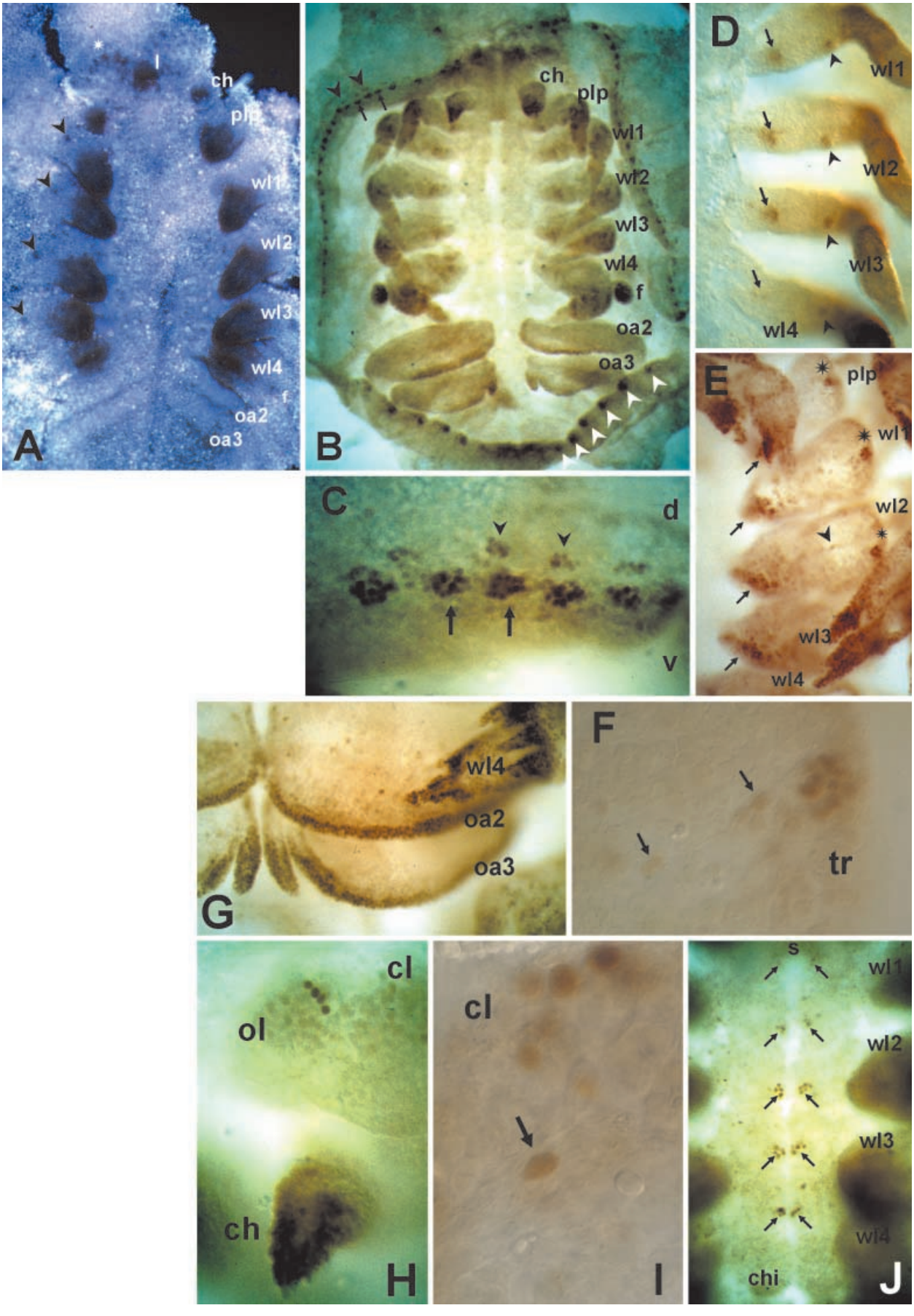
All these various sensory structures correspond almost exactly with areas of *Dll* expression described in the previous section.

Limulus polyphemus

Antibody staining

The embryos of *Limulus polyphemus* show a *Dll*-staining pattern that has not been described in other arthro-

Fig. 3A–J *Dll* expression in different stages of *Limulus polyphemus*. **A** Early embryo. All prosomal appendages express *Dll* at their tips. The *black arrowheads* mark the transient expressions, which are situated serially in a similar position as the *Dll*-positive flabellum (*f*) of the fourth walking leg. Many cells of the developing brain (star) are *Dll* positive. We use the term "pedipalp" for the second appendage of the horseshoe crab, because it only has the function and the shape of a normal walking leg in females; in adult males, these appendages are differentiated as claspers, which are used to grab the females at the opisthosoma for mating. The first pair of opisthosomal appendages, the chilaria (see **J**), appears later in development. **B** Overall view of a late embryonic stage. The whole prosomal part of the embryo is surrounded by two rows of *Dll*-expressing cell clusters (*arrows*, *black arrowheads*). On each side of the opisthosomal shield, there are six *Dll*-positive buds (*white arrowheads*). The distal part of all prosomal appendages and the edges and median processes of the opisthosomal appendages (*oa1*, *oa2*) express *Dll*. The flabellum shows a strong antibody staining. **C** Detail of the edge of the prosomal shield. The bigger *Dll*-positive cell clusters consists of 8–12 cells (*arrow*), the smaller ones of approximately 5 cells (*arrowheads*). **D** The four walking legs. Two cell clusters of the basal part of the limbs and pedipalps (not shown) express *Dll*: one in the coxa (*arrows*) and one in the adjointed trochanter (*arrowheads*).The coxal expression of the fourth walking leg (*wl4*) seems to be weaker than the other ones. **E** Limb bases of a later embryo. In the gnathobases of the pedipalps and all walking legs (*wl1–wl4*) extensive areas express *Dll* (*arrows*). Furthermore, one cluster of nerve cells of the trochanter (*stars*) is *Dll* positive. The *arrowhead* marks *Dll*-expressing cells, which accompany the nerve fibers inside the appendages; the nerve fibers of the remaining appendages are out of focus. **F** *Dll*-positive nerve cells in the trochanter. *Dll*-positive nerve cells (*arrows*) accompany the nerve fibers that connect the *Dll*-expressing nerve cells of the trochanter and the cell cluster of the coxa. **G** Opisthosomal appendages. The edge of the second opisthosomal appendage (*oa2*, the later operculum), the first gill-bearing opisthosomal appendage (*oa3*) and the median process of the latter show a distinct *Dll* expression. Furthermore, the four tarsal processes of the later "ski stick" of the fourth walking leg are *Dll* positive. **H** Developing brain. In the developing brain, several nerve cells in the optical lobe (*ol*) and the adjointed "cerebral" lobe (*cl*, out of focus) express *Dll*. **I** *Dll*-positive nerve cells of the cerebral lobe. **J** Central nervous system. Several cells of the developing ganglia show a distinct iterated *Dll* staining pattern (*arrows*). *ch* Chelicera, *chi* chilaria, *d* dorsal, *plp* pedipalp, *s* stomodaeum, *v* ventral


A
l
ch
plp
wl1
wl2
wl3
wl4
f
oa2
oa3
B
ch
plp
wl1
wl2
wl3
wl4
f
oa2
oa3
C
d
v
D
wl1
wl2
wl3
wl4
E
plp
wl1
wl2
wl3
wl4
F
tr
G
wl4
oa2
oa3
H
cl
ol
ch
I
cl
J
s
wl1
wl2
wl3
wl4
chi

pods. The whole embryo is surrounded by cell clusters expressing *Dll* (Fig. 3A). On the edge surrounding the prosoma, there are two rows of regularly arranged *Dll*-positive cell clusters. Each of the smaller clusters consists of approximately 5 cells, the large ones of 8–12 cells (Fig. 3C). In late embryonic stages, the whole area of the later dorsal side of the embryo is spotted by single cells showing distinct *Dll* expression (data not shown). On the edge surrounding the opisthosoma, there are six smaller buds on each side that express *Dll* at their tips (Fig. 3B). Apart from the chilaria, where we found several *Dll*-positive cells only in late stages, all prosomal appendages express *Dll* in a distal domain, which seems to begin at the femur. During further development, the chelae of the tarsi and the chelicera show a distinct staining in all prosomal appendages. Furthermore, the four tarsal processes of the fourth walking leg, which can be used by the animals while walking on the sand like a ski stick in the snow, are strongly expressing *Dll* (Fig. 3G). In addition, above this distal area, there are two more regions where the gene is active. In the most proximal part of the prosomal appendages, the basis, we found one cluster of cells expressing *Dll* that reaches a considerable extension in later stages (Fig. 3D, E). This expression seems to be weaker in the fourth walking leg. Furthermore, one group of nerve cells in the distal part of the adjointed trochanter expresses *Dll* (Fig. 3D–F). These groups of *Dll*-expressing cells are both connected by nerve fibers, that are accompanied by *Dll*-positive cells (Fig. 3E, F). The flabellum, probably a chemoreceptor which evolved from an epipodite of the fourth walking leg, also expresses *Dll* throughout its whole development. Interestingly, we found transient *Dll* expression without any outgrowth in the remaining prosomal appendages (except the chelicera) at the same position (Fig. 3A). This expression can be interpreted as remnants of former epipodites, that occur, for instance, in trilobites.

The outgrowth of the opisthosomal appendages (except the chilaria) is preceded by *Dll* expression in the shape of several pairs of narrow horizontal stripes with a width of a few cells (Fig. 3A). The slim edges of the semicircular buds of the later stages also show *Dll* expression (Fig. 3G). In addition, the tips of the paired median processes are *Dll* positive (Fig. 3G). It has been suggested that these processes are homologous to the endopods of stenopodous limbs (Walossek and Müller 1998). The median processes of the third opisthosomal appendage, the first appendage with book gills, develop earlier and reach a larger size than those of the operculum. Over a long period, a stronger *Dll* expression is visible on the median edge of the operculum and the posterior buds.

During the development of the central nervous system, there is also some evidence for participation of *Dll*. We found several *Dll*-positive nerve cells in the lateral optical lobes (Fig. 3A, H) and in the more medially situated "cerebral" lobes (Fig. 3J). In addition, some cells of the segmental ganglia show a distinct staining pattern (Fig. 3I).

Scanning electron microscopy

The hatching stage of *Limulus polyphemus* is called the trilobite stage because of its superficial similarities with this extinct arthropod group. Examinations of the trilobite stage with scanning electron microscopy provided a correspondence between the position of different sense organs, especially mechanoreceptors, and that of *Dll*-expressing cells in earlier stages just as in the silverfish. The whole prosoma of the trilobite stage is covered with small bristles. Along the edge of the shield surrounding the prosoma, there are two rows of different mechanoreceptors which are regularly arranged: on the dorsal side of the edge, there is a row of movable jointed bristles (Fig. 4A), while the ventrally located mechanoreceptors are peg sensilla (Fig. 4A, B). The cuticular peg sensillum consists of a hard peg with a small pore at the tip and is located on a cone of flexible cuticle. This flexible cuticle merges into the surrounding shield. The peg sensillum is the most ubiquitous mechanoreceptor of the horseshoe crab (Kaplan et al. 1976) and is distributed with variable density over the whole surface of the animal, in particular, along edges, ridges and spines (Fahrenbach 1999). It varies remarkably in size.

The basal parts of the prosomal appendages of *Limulus polyphemus* form gnathobases, which are used to process and transport food. Each gnathobase is equipped with numerous movable spines (Fig. 4C) and chemoreceptors (Barber 1956;Hayes and Barber 1967). The mouth of the horseshoe crab lies directly between the legs. If food touches this area, the horseshoe crab immediately starts to grab it with the legs and stuff it into its mouth; the stuffing is supported by alternating movements of the gnathobases, while the spines are also working as barbs (unpublished observation). The gnathobase of the fourth walking leg has only a small number of spines, approximately two to five. In the trilobite stage, these gnathobasic movable spines are not very numerous, because horseshoe crabs start feeding only after the following moult. In this advanced stage, the number of spines has increased remarkably and the spines are much larger. There are two or three movable jointed spikes in the distal area of the trochanter. During postembryonic development, they increase their number to four, and sometimes seven, spikes along the whole trochanter. On the edge of the opisthosoma, the six lateral spines are highly innervated. These spines are mechanoreceptors and each of them contains about 300 peg sensilla (Fig. 4D; Fahrenbach 1999). The edges of the semicircular gill bearing opisthosomal appendages and the operculum are densely covered with bristles (Fig. 4E, F).

Discussion

Our data show once again that *Dll* has additional functions beside participation in the development of extremities and their derivatives. Considering the invari-

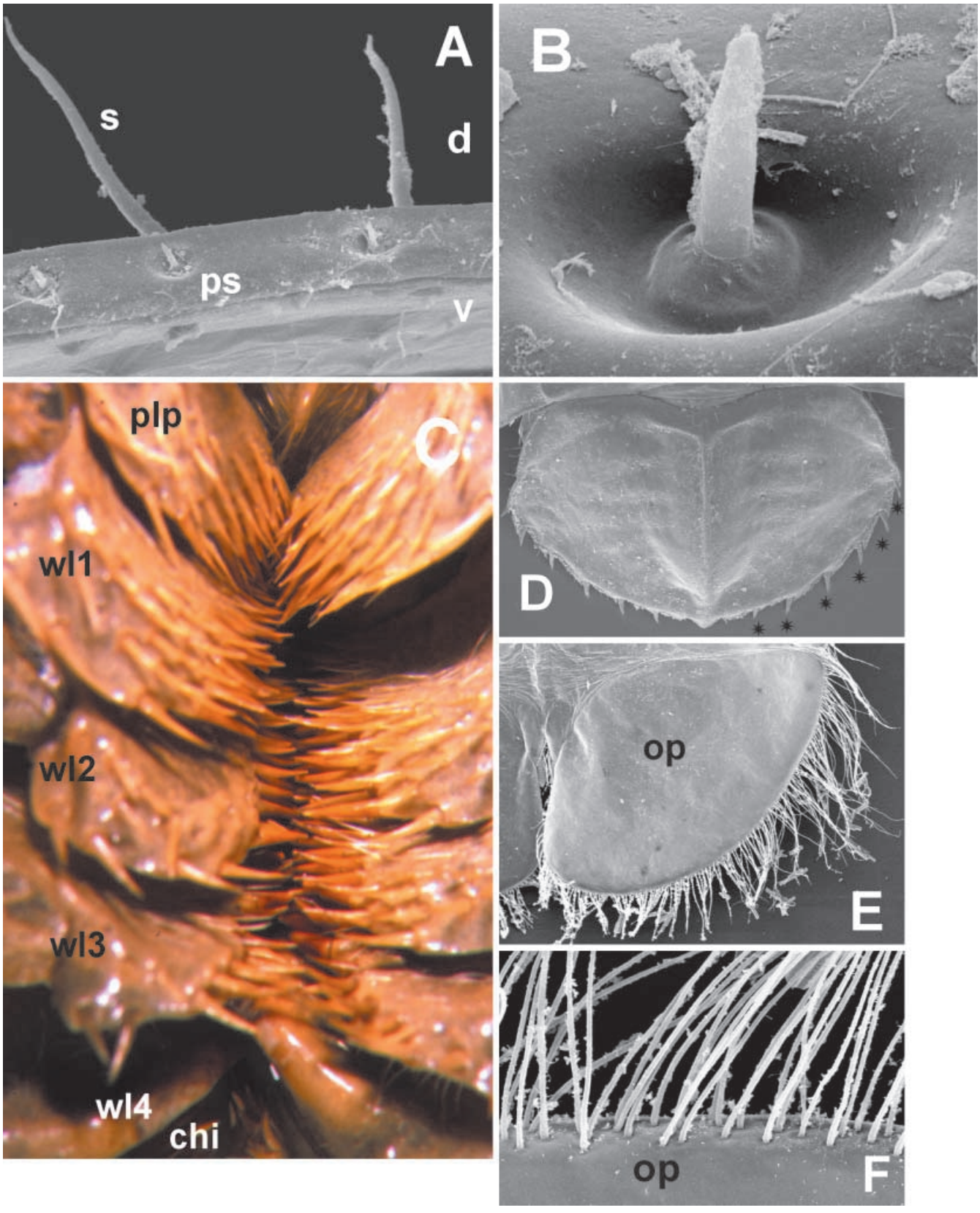


Fig. 4A–F SEM of larvae of *Limulus polyphemus*. **A** The edge of the prosomal shield. One row of setae (*s*) is situated along the dorsal side (*d*) of the prosomal shield edge, one row of peg sensilla (*ps*) along the ventral side (*v*). **B** Peg sensillum. **C** Stereomicroscopic image of the gnathobases of a several-years-old horseshoe crab. The whole coxal gnathobases of the pedipalps (*plp*) and the walking legs (*wl*), except the fourth one, are covered with movable jointed spines. Also, the chilaria (*chi*) show numerous spines. **D** Dorsal view of the opisthosoma of the trilobite stage. On each side of the opisthosomal shield, there are six movable spikes (*stars*). **E** Operculum of the trilobite stage. The whole edge of the operculum (*op*) is covered with long bristles. On the left side, the small median processes are visible. **F** Detail of the edge of the operculum

able and distinct *Dll* expression of particular cells in *Lepisma saccharina* and *Limulus polyphemus*, it is extremely improbable that these expressions are nothing more than accidental remnants of an earlier gene activity. One exception could be the proximal expression of the maxillary and labial palps: this ring-like expression near the transition of the second and third segment seems to be weaker than the distinct stainings in the other *Dll*-positive cells and shows some similarities to proximal expression in the legs of *Gryllus bimaculatus* (Niwa et al. 1997), *Lepisma saccharina* and several species of the Collembola (Mittmann, unpublished data). This expression could be caused by a requirement of the *Dll* product for the expression of other appendage-specific genes like *dachshund* (*dac*), *spalt* (*sal*), *aristaless* (*al*) or others (Panganiban 2000) or by the role of *Dll* in preventing cell mixing (Wu and Cohen 1999). The comparison of the antibody staining and the SEM observations in the remaining structures, however, provided remarkable correspondence between the position of mechanoreceptors or other sense organs and *Dll*-expressing cells. In addition to the *Dll* expression in nerve cells of the developing brain, the ganglia and prosomal legs, this correlation leads to the conclusion that this late expression demonstrates a requirement for the gene product during the development of particular structures of the nervous system and of sense organs. In the adult legs of *D. melanogaster*, *Dll* expression is restricted to bristles (Gorfinkiel et al. 1997) and the tissue fails to differentiate bristles in particular *Dll* mutants (Campbell and Tomlinson 1998). In the lethal null mutation of *D. melanogaster*, moreover, the larval peripheral sensory structures such as the antennal, maxillary or labial sense organs are missing (Cohen and Jürgens 1989a). We therefore conclude that the correspondence between *Dll* expression mentioned above and the position of setae or other sense organs in the labrum, mandibles, antennae, cerci and terminal filament, anal valves, lateral and ventral plates, etc. are caused by the differentiation of neurons or glia cells. Differences in the exact position of the setae and *Dll*-positive cells could be caused by cell migration, which has been presumed for several *Dll*-positive cells in the mite *Archegozetes longisetosus* (Thomas and Telford 1999), or by cell number increase.

Another indication of the supposed correlation is given by the second segment of the antenna, the pedicellus, which shows a clearer staining than the remaining segments; the pedicellus contains the Johnston's organ, a specific mechanoreceptor (chordotonal organ), that measures the position of the antenna. In the maxillary palps, *Dll*-expressing cells and receptors are found in similar areas, but we cannot determine with the methods used here whether the *Dll*-positive cells share their positions with later developed mechanoreceptors or chemoreceptors.

Apart from these rather obvious correspondences, several of our results give rise to some difficulties in the interpretation. An example is the expression pattern in the four lobes of both pairs of maxillae (glossa, paraglossa, galea, lacinia). These lobes are regarded as gnathobasic endites (Heymons 1897; Boudreaux 1987). They are thus parts of the coxopodites, which, together with the *Dll* expression in the endites of crustaceans and insects (Panganiban et al. 1995; Niwa et al. 1997; Rogers and Kaufman 1997; Scholtz et al. 1998; Williams 1998), disproves that only the telopodite expresses *Dll* (González-Crespo and Morata 1996; Niwa et al. 1997). However, the function of this particular gene activity is not obvious. On one hand, the budding itself could be the reason for the expression, consistent with the idea that the expression of *Dll* is possibly required for most budding events, whether they take place in appendages and related structures or not (Panganiban et al. 1997). On the other hand, *Dll* activity could be related to the development of the numerous sensilla trichodea which are found on both processes of the first and second maxillae (Larink 1978). We will be faced with the same problem when interpreting the expression pattern of several structures of the horseshoe crab.

Generally, the data from the horseshoe crab *Limulus polyphemus* support our idea that particular *Dll* expression is caused by a participation of the gene in the development of several mechano- or chemoreceptors. Most striking is the *Dll* expression in the prosomal legs, where nerve cells in the trochanter and cells along the nerve fibers that connect both clusters, show a distinct gene activity. In these cases, a neuronal function of *Dll* is obvious. Different interpretations are possible for the expression pattern in the gnathobases of the prosomal limbs. According to Snodgrass (1952) and several other authors, the part of the gnathobase bearing the spines is a coxal endite. We are thus faced with the same question, whether the budding itself or the development of neuronal structures (required for the innervation of the numerous movable spines) is responsible for the *Dll* expression. However, in the case of *Limulus*, the neuronal interpretation seems to be more obvious, because of a similar, strong innervation of the spines in the gnathobases and in the trochanter (Hayes and Barber 1967). Over and above that, the expression in the gnathobases of the fourth walking leg is somewhat weaker than in the other ones; this gnathobase is the only one which bears just a few spines in the larvae as well as in the adults. Furthermore, the chilaria, which are also covered with spines, show the first *Dll* expression some time after the budding event, so this expression pattern is rather like that of the silverfish mandible.

The bristles on the prosoma and especially the peg sensilla near the edge of the shield provide a striking correlation not only between the positions of *Dll*-expressing cell clusters and later appearing peg sensilla but also between the number of *Dll*-positive cells in each cluster and the number of neurons which contribute to a peg sensillum. Each sensillum consists of 8–10 neurons (Kaplan et al. 1976), and each cell cluster contains 8–12 *Dll*-expressing cells. However, in the case of the opisthosomal spines, operculum, and bookgill-bearing opistho-

somal limbs, the relation between *Dll* expression and sense organs is not as obvious as in the structures mentioned above. Anterior of each opisthosomal spine, there is a process which is part of the shield. Therefore, the expression could be caused by the outgrowth of this bud. On the other hand, it is also possible that the development of the spines themselves, which are highly innervated mechanoreceptors and covered by about 300 peg sensilla (Fahrenbach 1999), is correlated with *Dll* expression. To decide this question, further investigations are required, especially of sections of *Dll*-stained embryos and larvae. In the gill-bearing opisthosomal appendages, moreover, the early premorphogenetic expression in the shape of narrow stripes could be required for outgrowth of the semicircular buds or could be caused by the development of the numerous long bristles at the edge of the limbs. Interestingly enough, Popadic et al. (1998) and Abzhanov et al. (1999) could not find *Dll* expression in the booklungs and the lung covers of spider embryos until a late embryonic stage, even though they are thought to be homologous structures to the bookgills of merostomes, which show a very early expression in the horseshoe crab.

Even though the prosomal legs of *Limulus polyphemus* show two expression domains of *Dll*, there are still differences from the late expression patterns in the legs of *D. melanogaster*, *Gryllus bimaculatus* and apterygote insects (Panganiban et al. 1994; Gonzáles-Crespo and Morata 1996; Niwa et al. 1997; Mittmann, unpublished observations). All arthropods studied so far, including the horseshoe crab, share a distal expression of *Dll* in their legs. However, we never observed a ring-like expression in the trochanter and parts of the femur of *Limulus polyphemus*, but an extended *Dll*-positive area in the gnathobases; in the trochanter, just one distally situated cluster of nerve cells showed expression of the gene. Because a comparable striped pattern was never observed in other chelicerates, crustaceans or myriapods (Grenier et al. 1997; Popadic et al. 1998; Abzhanov et al. 1999; Thomas and Telford 1999; Abzhanov and Kaufman 2000), this expression pattern seems to be a specific character of insects.

Beside the direct activity of *Dll* in the brain development of representatives of insects and chelicerates and the developing central nervous system and nerve cell clusters in the prosomal legs of the horseshoe crab, our data show a striking correlation between the position of *Dll*-expressing cells in the embryos and the existence of several receptors, first and foremost mechanoreceptors, in the larvae and adult animals. They can be regarded as strong evidence that *Dll* participates in the differentiation of sense organs in arthropods in general. In future, we should control any *Dll* expression, which is not obviously required for the “classical” appendage development, to find whether it could be caused by a neurospecific function of that multifunctional gene.

Some speculations concerning the evolution of locomotory limbs

Over the course of the last few years, *Dll* has been detected in more and more representatives of the animal kingdom, from the nematode *Caenorhabditis elegans* and a polychaete annelid to several arthropods and vertebrates (Panganiban et al. 1995, 1997; Lowe and Wray 1997). The expression of the orthologue *Dll*/*Dlx* genes in the annelid parapodia, the lobopodia of onychophorans, the extremities of arthropods, the echinoderm tube feet, the fins of the zebrafish or the limb buds of mice is amazing; nevertheless these organs are not homologous structures (see also Tabin et al. 1999). From the expression patterns we can draw the conclusion that *Dll* must be an evolutionarily old gene, which evolved during an early period of the long history of animals. But this idea gives no satisfying explanation for the evolution of the function of the gene. Considering the expression pattern in detail, we find at least three separate processes in which *Dll* is involved: the specification of the animal pole during egg cleavage (Lee and Jacobs 2000); the development of extremities and their derivatives; and the development of the nervous system. Because all animals examined express *Dll*/*Dlx* in the nervous system, it has been speculated that one ancestral function of *Dll* could be its participation in the developing nervous system (Panganiban et al. 1997). As our data show, *Dll* function might not have been restricted to the CNS but might also have played an important role in differentiating sense organs. This ancestral neurogenetic function has been suggested for several other developmental genes involved in morphogenesis, including the Hox genes, which led to the hypothesis of a neuronal zootype (Deutsch and Le Guyader 1998). There is good reason to assume that the bilaterian stem species used most developmental genes primarily for the establishment of a proper central and peripheral nervous system. The problem is, however, how to explain the functional shift of genes or even gene complexes. There is little evidence to assume that the bilaterian stem species was equipped with any sort of locomotory or sensory appendage (Ax 1995). This makes it unlikely that *Dll* was already involved in the formation of body outgrowth of any kind in the urbilaterian. If, however, *Dll* was originally used to differentiate sense organs, the starting point for the formation of appendages in the various bilaterian lineages could have been the need for a higher spatial sensory resolution which can be achieved by outgrowths containing groups of sense organs. These outgrowths developed without *Dll* being involved in the budding process itself. Since there are body outgrowths which are formed without *Dll* activity this scenario seems plausible (Scholtz et al. 1998; see also Budd 1999 for a general discussion of the correlation between gene expression and evolution of morphogenesis). Later, the *Dll* gene was employed in appendage formation in several bilaterian lineages independently. The likeliness for this is even higher if one considers the putative *Dll* function in defining the animal-vegetal axis

in bilaterian eggs which requires similar mechanisms as defining the proximo-distal axis of limbs (Minelli 2000) and which also might have already occurred in the ancestor of recent Bilateria (Lee and Jacobs 2000). In summary, it seems plausible that locomotory limbs evolved as sensory outgrowths that became secondarily involved in locomotion of bilaterians. The combined function of differentiating sensory structures and the body axis made *Dll* a good candidate to be employed independently in limb formation in several bilaterian lineages.

Acknowledgements We thank Robert B. Barlow (Woods Hole, Syracuse) for his kind help to collect the eggs of *Limulus polyphemus* near Woods Hole, which took an enormous amount of time. The *Distal-less* antibody was a generous gift from Grace Panganiban. The comments of Graham Budd helped to improve the manuscript. Siegfried Rogaschewski (Institut of Physics, Humboldt-Universität, Berlin) spent a lot of time with us at the SEM. This work was supported by the Deutsche Forschungsgemeinschaft (Scho442/7–1).

References

Abzhanov A, Kaufman T (2000) Homologs of *Drosophila* appendage genes in the patterning of arthropod limbs. Dev Biol 227:1–17

Abzhanov A, Popadic A, Kaufman TC (1999) Chelicerate *HOX* genes and the homology of arthropod segments. Evol Dev 1:77–89

Adel T (1984) Sensilleninventar und Sensillenmuster auf den Antennen von *Thermobia domestica* und *Lepisma saccharina* (Insecta: Zygentoma). Braunschw Naturkd Schr 2:191–217

Ax P (1995) Das System der Metazoa I. Fischer, Stuttgart

Barber SB (1956) Chemoreception and proprioception in *Limulus*. J Exp Zool 131:51–74

Boudreaux HB (1987) Arthropod phylogeny with special reference to insects. Krieger, Malabar, Fla.

Budd GE (1999) Does evolution in body patterning genes drive morphological change – or vice versa? BioEssays 21:326–332

Campbell G, Tomlinson A (1998) The roles of the homeobox genes *aristaless* and *Distal-less* in patterning the legs and wings of *Drosophila*. Development 125:4483–4493

Cohen SM, Jürgens G (1989a) Proximo-distal pattern formation in *Drosophila*: cell autonomous requirement for *Distal-less* gene activity in limb development. EMBO J 8:1046–1055

Cohen SM, Jürgens G (1989b) Proximo-distal pattern formation in *Drosophila*: graded requirement for *Distal-less* gene activity in limb development. Roux's Arch Dev Biol 198:157–169

Cohen SM, Brönner G, Küttner F, Jürgens G, Jäckle H (1989) *Distal-less* encodes a homeodomain protein required for limb development in *Drosophila*. Nature 338:432–434

Deutsch J, Le Guyarder H (1998) The neuronal zootype. An hypothesis. C R Acad Sci Paris Sci Vie 321:713–719

Dong PDS, Chu J, Panganiban G (2000) Coexpression of the homeobox genes *Distal-less* and *homothorax* determines *Drosophila* antennal identity. Development 127:209–216

Fahrenbach WH (1999) Merostomata. In: Harrison FW, Foelix RW (eds) Chelicerate arthropods. (Microscopic anatomy of invertebrates, vol 8a) Wiley-Liss, New York, pp 21–115

Gonzáles-Crespo S, Morata G (1996) Genetic evidence for the subdivision of the arthropod limb into coxopodite and telopodite. Development 122:3921–3928

Gorfinkiel N, Morata G, Guerrero I (1997) The homeobox gene *Distal-less* induces ventral appendage development in *Drosophila*. Gen Dev 11:2259–2271

Grenier JK, Garber TL, Warren R, Whitington PM, Carroll S (1997) Evolution of the entire arthropod *Hox* gene set predated the origin and radiation of the onychophoran/arthropod clade. Curr Biol 7:547–553

Hayes WF, Barber SB (1967) Proprioceptor distribution and properties in *Limulus* walking legs. J Exp Zool 165:195–210

Heymons R (1897) Entwicklungsgeschichtliche Untersuchungen an *Lepisma saccharina* L. Z Wiss Zool 62:583–631

Kaphingst K, Kunes S (1994) Pattern formation in the visual centers of the *Drosophila* brain: *wingless* acts via *decapentaplegic* to specify the dorsoventral axis. Cell 78:437–448

Kaplan E, Barlow RB, Chamberlain SC, Stezner DJ (1976) Mechanoreceptors on the dorsal carapace of *Limulus*. Brain Res 109:615–622

Larink O (1978) Sensillenmuster auf dem Labium von Lepismatiden (Insecta: Zygentoma). Zool Anz 201:341–352

Larink O (1982) Das Sensillen-Inventar der Lepismatiden (Insecta: Zygentoma). Braunschw Naturkd Schr 1:493–512

Larink O (1983) Embryonic and postembryonic development of Machilidae and Lepismatidae (Insecta: Archaeognatha et Zygentoma). Entomol Gen 8 :119–133

Lee SE, Jacobs DK (2000) Expression of *Distal-less* in molluscan eggs, embryos and larvae. Evol Dev 1:172–179

Lowe CJ, Wray GA (1997) Radical alterations in the role of the homeobox genes during echinoderm evolution. Nature 389: 718–721

Minelli A (2000) Limbs and tail as evolutionary diverging duplicates of the main body axis. Evol Dev 1:157–165

Mittmann B (2000) Die Keimstreifbildung und das Expressionsmuster des Homöobox-Gens *Distal-less* bei primär flügellosen Insekten (Collembola und Zygentoma). Sitzungsber Ges Naturf Freunde Berlin 38:93–103

Niwa N, Saitoh M, Ohuchi H, Yoshioka H, 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

Panganiban G (2000) *Distal-less* function during *Drosophila* appendage and sense organ development. Dev Dyn 218:554–562

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

Panganiban G, Sebring A, Nagy L, Carroll SB (1995) The development of crustacean limbs and the evolution of arthropods. Science 270:1363–1366

Panganiban G, Irvine SM, Lowe C, Roehl H, Corley LS, Sherbon B, Grenier JK, Fallon JF, Kimble J, Walker M, Wray GA, Swalla BJ, Martindale MQ, Carroll SB (1997) The origin and evolution of animal appendages. Proc Natl Acad Sci USA 94:5162–5166

Popadic A, Rusch D, Peterson BT, Kaufman TC (1996) Origin of arthropod mandible. Nature 380:395

Popadic A, Panganiban G, Rusch D, Shear WA, Kaufman TC (1998) Molecular evidence for the gnathobasic derivation of arthropod mandibles and for the appendicular origin of the labrum and other structures. Dev Genes Evol 208:142–150

Rogers BT, Kaufman TC (1997) Structure of the insect head in ontogeny and phylogeny: a view from *Drosophila*. Int Rev Cytol 174:1–84

Romeis B (1989) Mikroskopische Technik, 17 Aufl. Böck P (ed) Urban und Schwarzenberg, Munich

Scholtz G, Mittmann B, Gerberding M (1998) The pattern of *Distal-less* expression in the mouthparts of crustaceans, myriapods and insects: new evidence for a gnathobasic mandible and the common origin of Mandibulata. Int J Dev Biol 42:801–810

Snodgrass RE (1952) Arthropod anatomy. Comstock, Ithaca, NY, pp 20–40

Sunkel CE, Whittle JRS (1987) *Brista*: a gene involved in the specification and differentiation of distal cephalic and thoracic structures in *Drosophila melanogaster*. Roux's Arch Dev Biol 196:124–132

Tabin CJ, Carroll SB, Panganiban G (1999) Out on a limb: parallels in vertebrate and invertebrate limb patterning and the origin of appendages. Am Zool 39:650–663

Thomas RH, Telford M (1999) Appendage development in embryos of the oribatid mite *Archegozetes longisetosus* (Acari, Oribatei, Thrypochthoniidae). Acta Zool (Stockholm) 80:193–200

Vachon G, Cohen B, Pfeifle C, McMuffin ME, Botas J, Cohen SM (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

Walossek D, Müller KJ (1998) Cambrian 'Orsten'-type arthropods and the phylogeny of Crustacea. In: Fortey RA, Thomas RH (eds) Arthropod relationships. Chapman & Hall, London, pp 139–153

Williams TA (1998) *Distalless* expression in crustaceans and the patterning of branched limbs. Dev Genes Evol 207:427–434

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

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