

The evolutionary transformation of phyllopodous to stenopodous limbs in the Branchiopoda (Crustacea) - Is there a common mechanism for early limb development in arthropods?

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ABSTRACT Arthropods and in particular crustaceans show a great diversity concerning their limb morphology. This makes the homologization of limbs and their parts and our understanding of evolutionary transformations of these limb types problematical. To address these problems we undertook a comparative study of the limb development of two representatives of branchiopod crustaceans, one with phyllopodous the other with stenopodous trunk limbs. The trunk limb ontogeny of a 'larger branchiopod', *Cyclestheria hislopi* ('Conchostraca') and the raptorial cladoceran *Leptodora kindtii* (Haplopoda) has been examined by various methods such as SEM, Hoechst fluorescent stain and expression of the *Distal-less* gene. The early ontogeny of the trunk limbs in *C. hislopi* and *L. kindtii* is similar. In both species the limbs are formed as ventrally placed, elongate, subdivided limb buds. However, in *C. hislopi*, the portions of the early limb bud end up constituting the endites and the endopod of the phyllopodous filtratory limb in the adult, whereas in *L. kindtii*, similar limb bud portions end up constituting the actual segments in the segmented, stenopodous, and raptorial trunk limbs of the adults. Hence, the portions of the limbs corresponding to the endites of the phyllopodous trunk limbs in *C. hislopi* (and other 'larger branchiopods') are homologous to the segments of the stenopodous trunk limbs in *L. kindtii*. It is most parsimonious to assume that the segmented trunk limbs in *L. kindtii* have developed from phyllopodous limbs with endites and not *vice versa*. This study has demonstrated at least one way in which segmented limbs have been derived from phyllopodous, multi-lobate limbs during evolution. Similar pathways can be assumed for the evolution of stenopodous, segmented and uniramous limbs in other crustaceans. Irrespective of the differences in the adult limb morphology, the early patterning of arthropod limbs seems to follow a similar principle.

KEY WORDS: *Distal-less*, *Leptodora*, *Cladocera*, *phylogeny*, *embryology*, *ontogeny*

Introduction

Arthropods exhibit a great diversity in the morphology of their limbs. In addition to highly modified appendages used as sensory organs or as mouthparts, the trunk limbs which are involved in locomotion and in several instances in food transport vary to a high degree. One finds uniramous, biramous or polyramous legs, which are either of tube-like (stenopodous) or of flattened (phyllopodous) shape. In some cases there is only weak articulation within the limbs, in others there are distinct joints. Moreover, the number of limb segments varies between the arthropod taxa. This high degree of variety in limb shape can already be seen in Cambrian arthropod representatives (e.g., Gould, 1989; Walossek, 1993; Conway Morris, 1998; Budd, 1996). These morphological

differences of arthropod limbs have caused numerous investigations and speculations dealing with comparative aspects of limb development and evolution. In particular, the following questions are controversially discussed: are limb segments homologous between higher arthropod taxa (Hessler and Newman, 1975; Abzhanov and Kaufman, 2000), do polyramous phyllopodous limbs share similar developmental gene expression patterns and principles of axis formation with uniramous stenopodous limbs (Panganiban *et al.*, 1995; González-Crespo and Morata, 1996;

Abbreviations used in this paper: a1, antenna 1; a2, antenna 2; ca, carapace; Dll, *Distal-less*; e1-e5, endite 1-5; en, endopod; ep, epipod; ex, exopod; la, labrum; mx1, maxilla 1; mx2, maxilla 2; SEM, Scanning Electron Microscopy; tl1, trunk limb 1; tl6, trunk limb 6; tl7, trunk limb 7; tl10, trunk limb 10.

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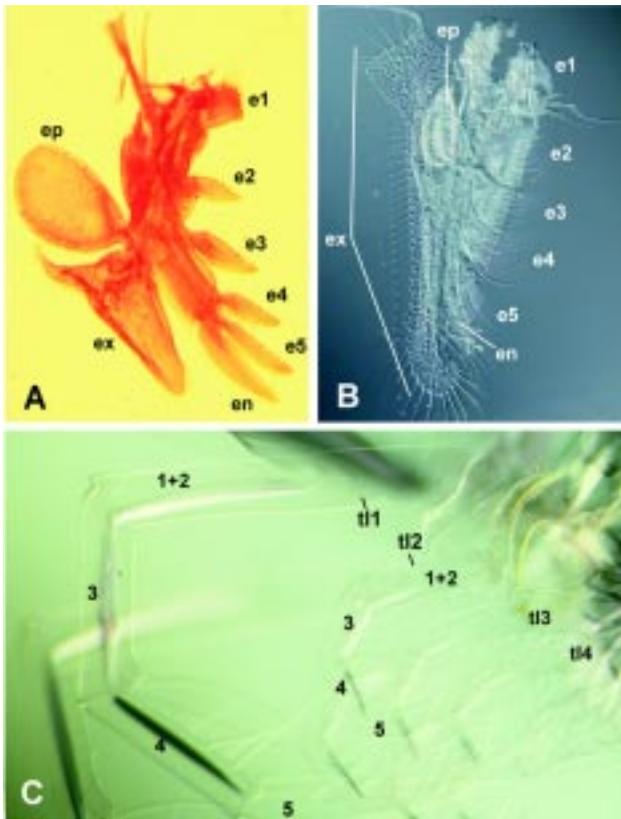


Fig. 1. Light microscopy of adult trunk limbs of two 'large branchiopods' and one predatory cladoceran to show diversity of trunk limbs within the Branchiopoda. (A) *Triops* sp. (preparation from the zoological collection of the Humboldt University). (B) *Cyclestheria hislopi*. (C) *Leptodora kindtii* (preparation from the zoological collection of the Humboldt University), lateral view of left side trunk limbs, the distal-most segment of trunk limbs 1 (left) is not shown. Abbreviations: e1-e5-endite 1-5; en, endopod; ep, epipod; ex, exopod; tl1-4, trunk limbs 1-4; arabic numbers 1-5 refer to embryonic limb portions in *L. kindtii*.

Williams, 1998, 1999; Nulsen and Nagy, 1999; Nagy and Williams, 2001), are phyllopodous limbs plesiomorphic within arthropods or subgroups like Crustacea or did phyllopodous limbs evolve several times independently from stenopodous appendages (Lauterbach, 1978; Martin and Christiansen, 1995; Spears and Abele, 1999; Ax, 1999), can insect wings be derived from epipodites (Kukalová-Peck, 1992; Averof and Cohen, 1997)?

Among the extant arthropod groups, the Crustacea shows the greatest diversity in trunk limb morphology and this high degree of diversity is also seen in each of the major crustacean taxa such as malacostracans or branchiopods. Examples of the latter are treated in the present paper. The trunk limbs of the 'larger branchiopods' (Anostraca, Notostraca and 'Conchostraca') are relatively similar. All have a high number of serially similar, more or less flattened trunk limbs (phyllopods), composed of a large corm (the 'basis', after Walossek, 1993), normally with 6 setose lobes (at least in adults) of variable shape along the inner margin, the proximal one normally with a differing morphology and directed into the food groove. Five of these lobes are usually considered as endites. However, the sixth, distalmost 'lobe', which in some cases looks like an endite, has by comparison to certain 'Orsten' fossils been suggested to be homologous to the

endopod of other Crustacea (Walossek, 1993). At the outer margin of the trunk limbs they normally have a setose exopod of variable shape and a non-setose, sac-like epipod (gill). In contrast to this, a much greater variation of trunk limb morphology is seen within the Cladocera where only 4 to 6 trunk limbs are present (Fryer, 1987; Dumont and Silva-Briano, 1998).

In this paper we focus on a raptorial cladoceran - *Leptodora kindtii* representing the monotypic Haplopoda - which is, due to its lifestyle as a predator, among the most deviating branchiopods. This cladoceran has uniramous, stenopodous, segmented trunk limbs, and any comparison between these and the flattened, multi-lobate limb type described above for the 'larger branchiopods' is difficult.

We examined embryonic stages of *Leptodora kindtii* (Haplopoda) and one 'larger branchiopod', *Cyclestheria hislopi*, the presumed sister group to the Cladocera, by different morphological methods. In particular, we used a polyclonal antibody against the *Distal-less* (*Dll*) gene product (Panganiban et al., 1995). The *Dll* gene has been shown to play an important role for arthropod limb development and its expression patterns have been used to infer on homology of limbs and their parts respectively (e.g., Panganiban et al., 1995; Niwa et al., 1997; Popadic et al., 1998; Scholtz et al., 1998; Williams, 1998; Thomas and Telford, 1999; Abzhanov and Kaufmann, 2000; Browne and Patel, 2000; Scholtz, 2001). Examination of embryos of *Cyclestheria hislopi* and various cladocerans has earlier proved useful when trying to establish homologies between taxa where the adults are difficult to compare (Olesen, 1998, 1999).

We address the following questions: What are the homologies between the segmented trunk limbs of the raptorial cladocerans and the typical 'multi-lobate', phyllopodous trunk limb of the 'larger branchiopods'? Based on this homologisation - what evolutionary scenario for the development of the segmented limbs can be outlined? Can the evolution of the branchiopod limbs serve as a model for arthropod limb transformation during development and evolution?

Results

The early development of the trunk limbs in *Leptodora kindtii* and *Cyclestheria hislopi* is quite similar. In both taxa they are formed as two ventral rows of elongate, subdivided limb buds, six pairs of limb buds in *L. kindtii* and 15-16 in *C. hislopi*. In *C. hislopi* the long limb buds first form a lateral tip, then they divide into six ventral portions (starting from proximal), attain a bifurcate tip (which appears before all ventral portions are formed), develop a primordial epipod which originates dorsal to the bifurcate tip, and then shift the orientation of the entire limb to an 'upright', vertical position (Figs. 2,3). *L. kindtii* has a similar tip formation and subdivision of the early, elongate limb bud as *C. hislopi*, but the number of separate portions of the limb bud is reduced to five, as revealed by SEM (Fig. 4), by Hoechst nuclear stain (Fig. 5) and by expression of the *Distal-less* (*Dll*) gene (Fig. 6). Different from the limb development in *C. hislopi* is that *L. kindtii* develops no bifurcate tip and no epipod. The early pattern of *Dll* expression is quite similar between *C. hislopi* and *L. kindtii*. The *Dll* expression in each limb is first seen in a lateral area, which later forms the tip of the appendage (Figs. 3,6). The second spot of *Dll* expression is found in the most proximal area of the forming limb (Figs. 3,6). In more advanced stages of both species a series of *Dll* positive

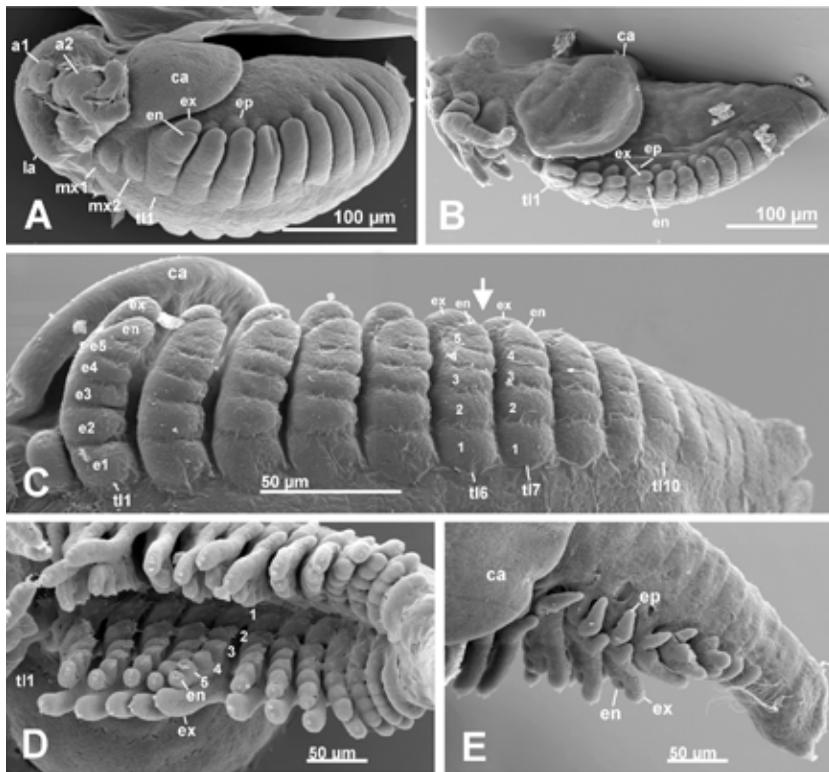


Fig. 2. Embryonic stages of *Cyclestheria hislopi* ('Conchostraca') (SEM). (A) Lateral view of stage IV. (B) Ventral view of stage VI. (C) Left side trunk limbs, ventral, stage VI. Fifth endite becomes separated from endopod between trunk limb 6 and 7 (white arrow). (D) Ventral view of trunk limbs of stage VII. (E) Lateral view of posterior trunk limbs of stage VII. Abbreviations: a1, antenna 1; a2, antenna 2; ca, carapace; la, labrum; mx1, maxilla 1; mx2, maxilla 2; tl1, trunk limb 1; e1-e5, endite 1-5; en, endopod; ex, exopod; tl6, trunk limb 6; tl7, trunk limb 7; tl10, trunk limb 10; ep, epipod.

regions appears at the upper margin of the limb anlage. In *C. hislopi* these expression areas correspond with the forming inner lobes. In *L. kindtii* the *Dll* positive regions are associated with the prospective limb segments (Fig. 6 C,D). It appears that *Dll* expression fades in the proximal limb segments of more advanced embryos of *L. kindtii*. In contrast *Dll* expression is maintained in the lobes of the *C. hislopi* limbs throughout the observed development (Fig. 3 C,D). No *Dll* expression has been found in the epipod. The way in which the limbs attain their vertical orientation is also different in *L. kindtii* and appears more complicated than in *C. hislopi*. Whereas *C. hislopi* effectively 'bends' the whole limb (Fig. 2), the trunk limbs of *L. kindtii* have a characteristic bend in the region of the first and

second segments in the intermediate to late parts of their ontogeny (Figs. 4,5).

The limb portions of the early limb bud of *C. hislopi* are simple to account for in terms of their further development. The five proximal ventral portions end up constituting endites 1-5 in the adult, while the distalmost sixth portion develops into the small unsegmented endopod (Figs. 2,3). The dorsal part of the bifurcated tip of the limb buds will constitute the exopod in the adult, while the dorso-laterally placed portion will constitute the epipod (see Figs. 1,2).

In *L. kindtii* the situation is more complicated and difficult to follow during the development, despite there are fewer limb portions to account for. Trunk limb 1-5 in the adults of *L. kindtii* have four segments each, one very long proximal segment followed by three smaller segments (see Sars, 1993). The elongate limb buds of early embryos consist of five portions as revealed by the expression of *Dll* (mentioned above, see Figs. 4-6). It is clear that the three distal (or lateral) portions of the early limb bud will end up constituting the three distal short segments present in trunk limb 1-5 in the adult, since the fate of these limb portions can be followed unambiguously during ontogeny (see Fig. 7). The fate of the two proximal portions seen in the early embryos, revealed by *Dll* expression, is more difficult to follow and, furthermore, the development of trunk limb 1 is slightly different from that of trunk limb 2-5. Common to trunk limbs 1-5 is that the large proximal segment in the adult arise from a fusion of two rudimentary embryonic portions indicated by the *Dll* expression (marked by Arabic numbers 1 and 2 on Figs. 4-6).

However, even more proximal, slightly 'bend' under the more distal part of the limb, is a limb portion which has not been *Dll* positive, but which nevertheless becomes included in the long proximal part of the adult trunk limb (Figs. 4 B,C, 5B, marked by upper arrow). This limb part is best visible in the anterior limb pairs. We interpret this limb portion as a part of embryonic limb

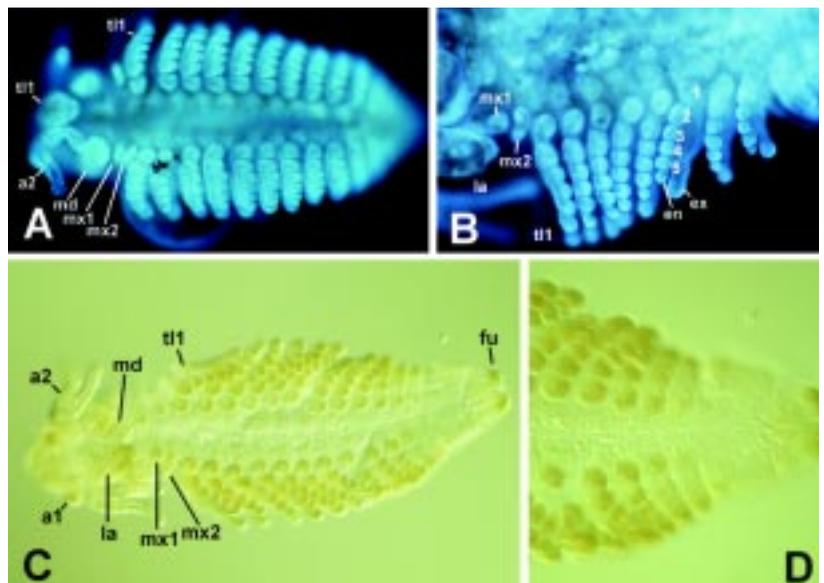


Fig. 3. Embryonic stages of *Cyclestheria hislopi* ('Conchostraca'). (A) Ventral view of *Cyclestheria hislopi*, intermediate stage (stage VI) (Hoechst nuclear stain). (B) Right side trunk limbs of late stage of *Cyclestheria hislopi* (stage VII) (Hoechst nuclear stain). (C) *Cyclestheria hislopi*, ventral view, stage VII (Distal-less immunostaining). (D) *Cyclestheria hislopi*, ventral view, posterior part (Distal-less immunostaining). Staging after Olesen (1999). Abbreviations: a1, antenna 1; a2, antennae 2; en, endopod; ex, exopod; fu, furcae; la, labrum; md, mandible; mx1, maxilla 1; mx2, maxilla 2; tl1, trunk limb 1; arabic numbers 1-5 indicate endites 1-5.

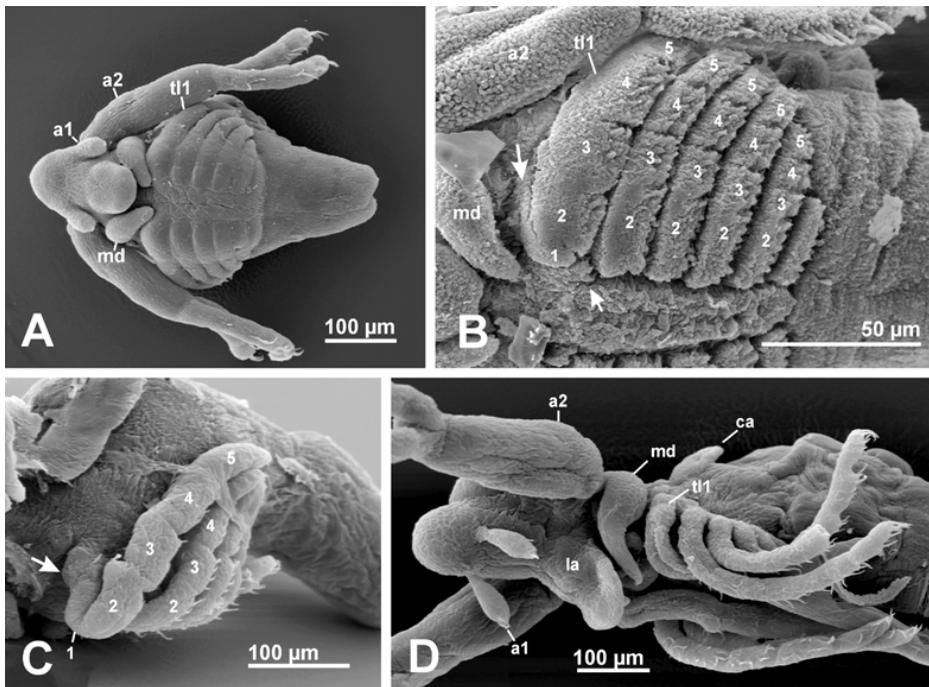


Fig. 4. Embryonic stages of *Leptodora kindtii* (SEM). (A) Ventral view of an intermediate stage 2. (B) Left side trunk limbs of an intermediate stage 2, upper arrow points to the 'folded' part of trunk limb 1, lower arrow points to the so-called 'maxillary process' (see text for details). (C) Left side trunk limbs of stage 3, arrow points to basal, folded part of first trunk limb. (D) Left side of stage 4 (juvenile/sub-adult). Abbreviations: a1, antenna 1; a2, antenna 2; md, mandible; tl1, trunk limb 1; la, labrum; ca, carapace. Arabic numbers 1-5 mark limb portions.

portion 1. In the adults, trunk limb 1 differs from the more posterior trunk limbs in the presence of a small, setose (two setae) so-called 'maxillary process' situated at the broad mid region between the limb, close to bases of the limbs, but not connected to these (e.g. Sebestyén, 1931). In early embryos, the expression of *Dll* in the proximal segment is located more closely to the midline in the first trunk limbs than in the more posterior ones (Fig. 6D). This median expression exactly corresponds to the *anlagen* of the 'maxillary process' (Fig. 5 B,C, lower arrow). We interpret this as indicating that the endite of the proximal portion of the limb migrates towards the midline of the animal, and ends up constituting the 'maxillary process'. Hence, the 'maxillary process' has nothing to do with the maxillae. The endites of the proximal parts of the subsequent trunk limbs remain associated with the corresponding limb portion.

Discussion

*Limb homologies in *Cyclestheria hislopi* and *Leptodora kindtii**

Since *Cyclestheria hislopi* is most likely the sister group to a monophyletic Cladocera (see Martin and Cash-Clark, 1995; Olesen et al., 1997; Olesen, 1999; Crease and Taylor, 1998; Ax, 1999; Taylor et al., 1999; Spears and Abele, 2000; Braband et al., 2000), there are good reasons for comparing the limb development and morphology of *L. kindtii* and *C. hislopi*.

As mentioned above, the pattern of subdivision of early limb buds in *C. hislopi* and *L. kindtii* is very similar. In contrast, the limbs continue their development in two completely different directions (summarised in Fig. 7). In *C. hislopi* the five proximal portions end up constituting the five endites present in the adult. The sixth portion ends up constituting the distal-most lobe on the inner corm of the limb, the endopod. Most other 'larger branchiopods' show a similar pattern of limb development as has been shown for the notostracan *Triops longicaudatus* (Williams and Müller, 1996)

and the anostracans *Branchipus stagnalis* and *Branchipus schaefferi* (Claus, 1873; Schlögl, 1996). Since this type of development is not found outside the Branchiopoda, it has been suggested as being a synapomorphy for the Branchiopoda (Olesen, 1999).

In *L. kindtii*, by contrast, the various portions of the early limb bud ends up constituting the actual segments in the stenopodous trunk limbs in the adult (see above for more details). Based on these ontogenetic findings, we propose the following homologies between *L. kindtii* and *C. hislopi* (and thereby other larger branchiopods). The long basal segment in *L. kindtii* is homologous to endites 1 and 2 and the corresponding parts of the limb corm in *C. hislopi*. This picture is most clear for trunk limbs 2-5. Associated to the first pair of trunk limbs in *L. kindtii* is a pair of so-called 'maxillary processes', placed on the ventral trunk area, disconnected from the actual limb. These are probably homologous to the proximal endites of the first trunk limbs in *C. hislopi*. The three smaller distal segments of the trunk limbs in *L. kindtii* correspond to endites 3-5 (including the corresponding parts of the limb corms) and the small, unsegmented endopod in *C. hislopi*. In *C. hislopi*, the ventral portions (future 5 endites, endopod and exopod) of the elongate limb bud are formed gradually from proximal to distal with the exopod and epipod formation sometime in between (Fig. 2C, see Olesen, 1999 for more details). This is reflected by the *Dll* expression, which is first seen in the distal tip of the limb anlagen, followed by the proximal endite, and subsequently more distal endites. The exopod and endopod originate from a bifurcation of the distal portion of the limb anlage. Except for some differences in the timing of the subdivision and the *Dll* expression a similar pattern of limb development has been described in the notostracan *Triops longicaudatus* (Williams, 1998). In *L. kindtii* only five limb portions are present and the two proximal parts fuse to a single segment. Therefore, the distal most limb segment corresponds not only to the fifth endite but also to the endopod of

C. hislopi. It is furthermore reasonable to assume that the absence of the exopod in the limbs of *L. kindtii* is the result of the suppressed bifurcation of the early distal limb portion.

Evolution of stenopodous trunk limbs in *Leptodora kindtii*

The homologies between the trunk limbs in *Cyclestheria hislopi* and *Leptodora kindtii* are established in some detail above and, broadly speaking, the endites including the corresponding part of the basis of *C. hislopi* and other larger branchiopods are homologous to the true segments in *Leptodora*. But which is derived from which?

The phylogenetic position of *L. kindtii*, as a member of the Cladocera within the Branchiopoda irrespective of its exact sister group relationship (Martin and Cash-Clark, 1995; Olesen *et al.*, 1997; Olesen, 1998; Crease and Taylor, 1998; Schwenk *et al.*, 1998; Taylor *et al.*, 1999; Negrea *et al.*, 1999; Spears and Abele, 2000; Richter *et al.*, 2001), indicates that the segmented limbs of this taxon have been derived from the typical larger branchiopod limb and not vice versa. If the segmented limbs of *L. kindtii* were viewed as homologous to the segmented limbs so commonly seen in other crustaceans, and therefore retained through the whole evolution of the Branchiopoda, it would imply convergent evolution of the typical flattened trunk limb a number of times within the 'larger Branchiopoda' and the Cladocera, which is therefore unrealistic. Hence, the similarity between the segmented limbs of *L. kindtii* and the similarly segmented limbs of so many other Crustacea is considered convergent.

This allows for a plausible evolutionary scenario where it is possible to account convincingly for functional intermediate stages between a filter feeding branchiopod, as represented by most 'larger branchiopods', with their flattened, endite bearing limbs and the raptorial feeding *L. kindtii*, with its uniramous, stenopodous, segmented limbs. During the evolution of the segmented limb of *L. kindtii* from flattened, endite bearing limb, as those of the 'larger Branchiopoda', one must imagine the following morphological changes. 1) Transformation of the clefts between the endites gradually developing into articulations. 2) Development of tubular articles of the limb and a more rigid cuticle. 3) Loss of exopod (possibly by suppression of the bifurcation of the tip of the limb during development) and epipod (not necessarily related to loss of exopod). 4) Fusion of regions corresponding to endite 1 and 2 to one long tubular segment. 5) Alteration of endite formation (possibly by ontogenetic suppression of subdivision) so that only five limb portions are formed.

The exact order in which these evolutionary changes took place, or to which extent the various steps could have been linked to each other, is not known for certain, but some suggestions can be made. One obvious possible correlation is that the appearance

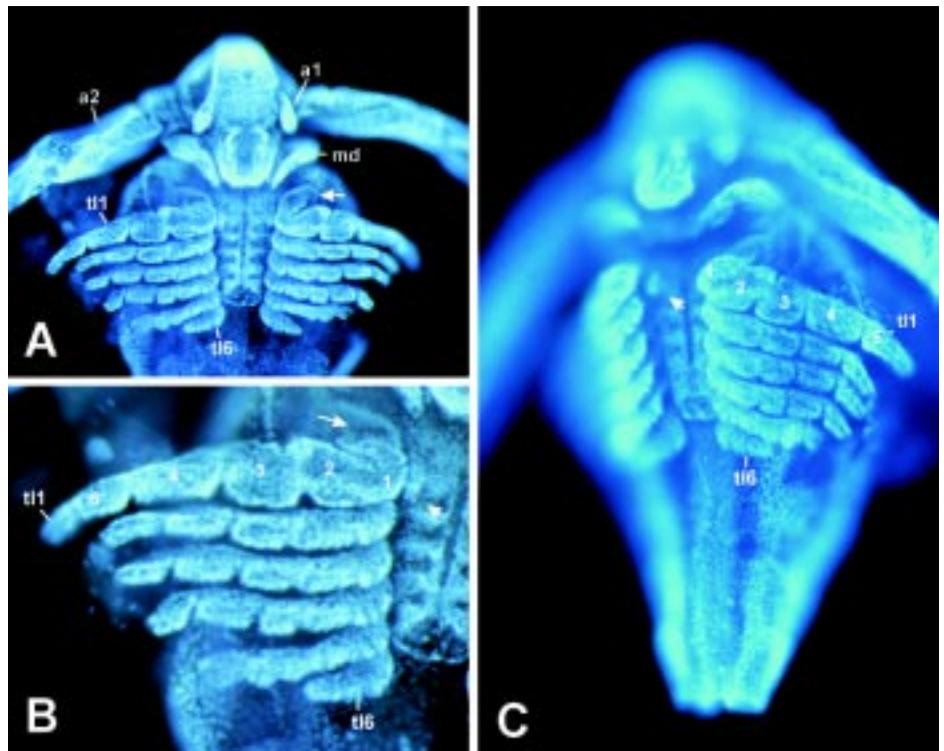


Fig. 5. Embryonic stages of *Leptodora kindtii* (Hoechst nuclear stain). (A) Ventral view of stage 2, arrow points to 'folded' part of trunk limb 1. (B) Close-up of A, upper arrow points to 'folded' part of trunk limb 1, lower arrow points to the so-called 'maxillary process'. (C) Late stage 2, arrow points to so-called 'maxillary process'. Abbreviations: a1, antenna 1; a2, antenna 2; md, mandible; t11, trunk limb 1; t16, trunk limb 6. Arabic numbers 1-5 mark limb portions.

of articulations (step 1) can be seen as linked to the appearance of a more rigid cuticle (step 2).

Two features are characteristic for the limbs of *L. kindtii* as compared to those of *C. hislopi* and other 'larger branchiopods', the fact that they are *stenopodous* and *uniramous*. While we cannot exclude that the appearance of these two characteristics have been evolutionary linked, we have, on the other hand, no reason to believe so.

Implications for limb evolution in the Crustacea

Already Borradaile (1926) speculated, based on comparative morphology, that the primitive crustacean limb was unsegmented but had endites from which true segments had developed. Also Fryer (1992) put forward the view that arthropod appendages had originated as unsegmented structures. Now it is clearly shown in the present paper that truly segmented uniramous limbs have evolved from phyllopodous, multi-lobate limbs at least once during crustacean evolution (within the Recent branchiopods). It is tempting to suggest that a similar scenario has occurred in the evolution of segmented limbs in other crustaceans and arthropods. Trunk limbs with a row of identical lobate endites - very similar and most likely homologous to those of Recent branchiopods - are a very old phenomenon, present already in the Upper Cambrian in certain crustacean 'Orsten'-fossils, like *Rehbachella kinnekullensis* Müller, 1983, *Walossekia quinquespinosa* Müller, 1983 and *Dala peilertae* Müller, 1983 (see Walossek, 1993, 1999). Accordingly, there is room for interpreting phyllopodous trunk limbs with such morphology as

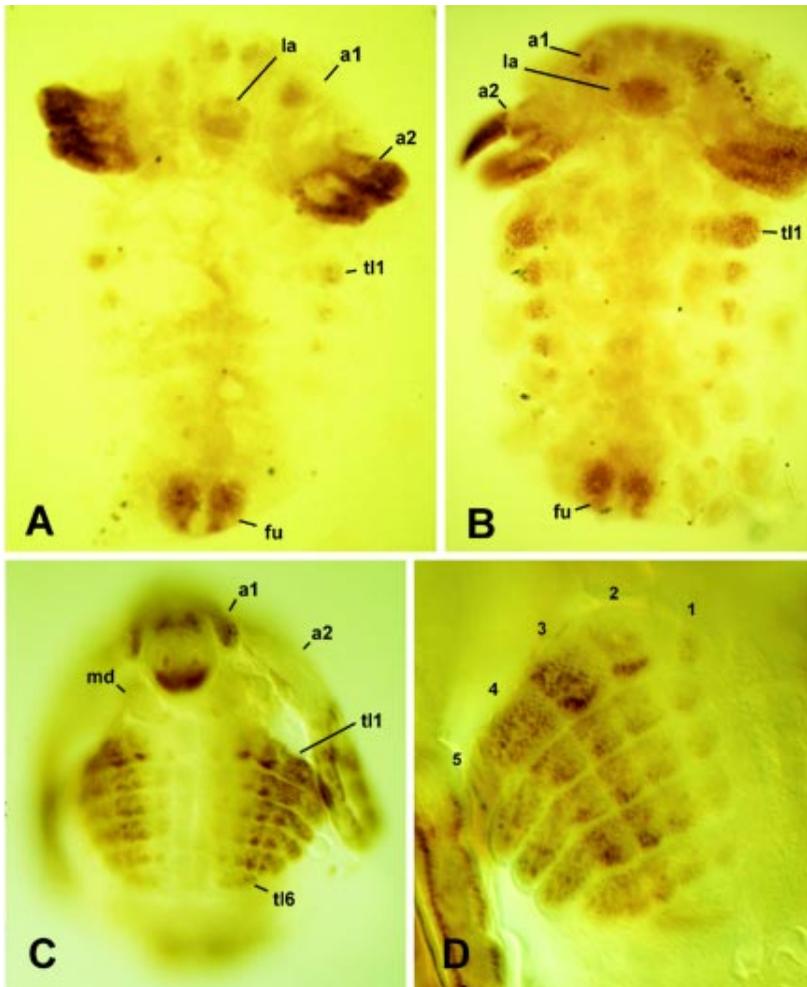


Fig. 6. Embryonic stages of *Leptodora kindtii* (Distal-less immunostaining). (A) Early stage 1. (B) Stage slightly later than A. (C) Late stage 1/early stage 2. (D) Close-up of C. Abbreviations: a1, antenna 1; a2, antenna 2; fu, furcae; md, mandible; tl1, trunk limb 1; tl6, trunk limb 6. Arabic numbers 1-5 mark limb portions.

ancestral also for other Recent Crustacean groups. We therefore suggest the transformation of limb endites into true segments, as a working hypothesis for the evolution of other Crustacea with segmented limbs. However, studies focussing on small but well-identified aspects of limb evolution, with high attention to segmentation homologies, much in the spirit of Boxshall and Huys (1992), are pivotal if we wish to understand such questions and the evolution of crustacean and arthropod appendages in general.

The best candidate for a similar scenario of limb transformation from phyllopodous to stenopodous would be the Malacostraca. Foliaceous trunk limbs are found in the malacostracan subgroup Leptostracana which is most likely the sister group to the remaining malacostracans with stenopodous limbs, the Eumalacostraca (Richter and Scholtz, 2001). Accordingly, it is reasonable to assume that the stenopodous segmented limbs of the Eumalacostraca have evolved from foliaceous limbs similar to those of Recent leptostracans. However, whether foliaceous limbs are really plesiomorphic for the Malacostraca depends on the homology between phyllopodous limbs of branchiopods and leptostracans which has been suggested, for instance, by Lauterbach (1975), Schram (1986), and Ax (1999). On the other hand, this homology

has been rejected by other authors (Dahl, 1987; Walossek, 1993; Martin and Christiansen, 1995; Spears and Abele, 1999).

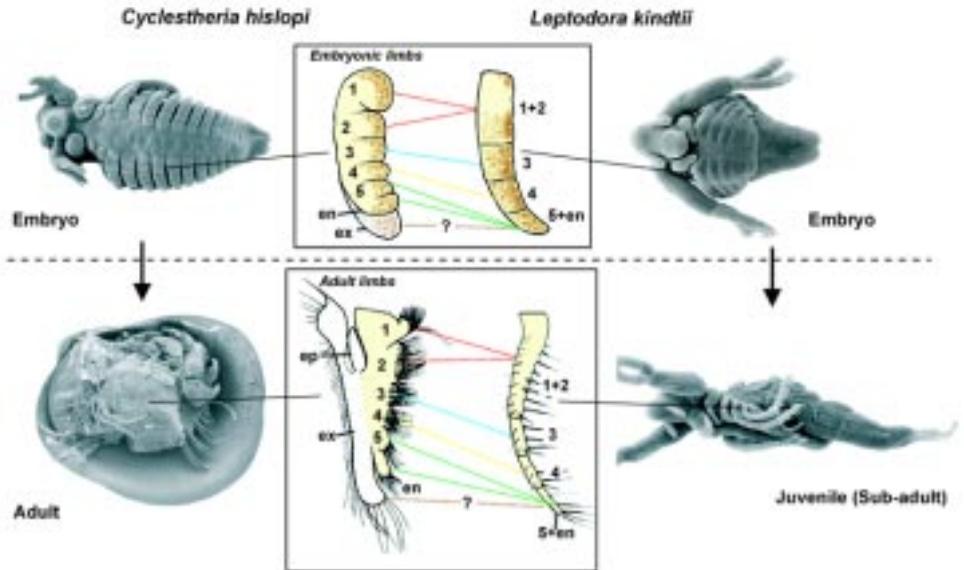
As a last remark, we find it interesting to note that Walossek's (1993) hypothesis about the origin of the crustacean coxa from an (proximal) endite receives some support from our results. The described transformation of endites into true limb segments within branchiopods are, at the general level, essentially the same thing as the transformation events (from the proximal endite to a coxa (limb segment)) hypothesised by Walossek (1993)

The P/D axis is the same in uniramous and polyramous arthropod limbs

Our data clearly show that similar limb anlagen in terms of morphology and expression of *Dll* can result in highly different multi-lobed, phyllopodous and stenopodous, uniramous limbs. This provides a solution to the problem of the comparability of the axis formation in uniramous and multilobed limbs raised and discussed by Williams and Nagy (1996), Williams (1999), Nulsen and Nagy (1999), and Nagy and Williams (2001). Williams (1999) discussed whether the phyllopodous limbs possess one P/D axis as the uniramous *Drosophila melanogaster* legs or whether each lobe of the branchiopod leg is equivalent to a *Drosophila* limb in terms of axis formation. Our results now directly support the conclusions by Williams and Nagy (1996), that the latter hypothesis is unlikely and polyramous limbs are evolutionarily transformed into uniramous limbs (and probably *vice versa*) without changes of the early axis determination as shown by the *Dll* expression. This is indicated by the fact that in all cases the earliest *Dll* expression is found in the prospective distal tip of the limb anlagen irrespective of their shape (Panganiban *et al.*, 1995; Niwa *et al.*, 1997; Williams, 1998; Scholtz *et al.*, 1998; Popadic *et al.*, 1998; Mittmann and Scholtz, 2001). This is in good agreement with the function of the *Dll* gene in the insect *Drosophila melanogaster* and in the spider *Cupiennius salei* where

Dll has been found to be responsible for differentiation of the P/D axis and the formation of the distal limb elements (Cohen and Jürgens, 1989; Schoppmeier and Damen, 2001). Interestingly enough, the second (in time) area of *Dll* expression is found in the most proximal part of the limb anlage - if an endite is at least transiently formed. This is again true for the anlagen of phyllopodous and stenopodous limbs and can be found even in hexapod mouthparts (Niwa *et al.*, 1997; Scholtz *et al.*, 1998). This sequence of limb differentiation starting with the distalmost elements followed by the most proximal ones is also found in the morphological differentiation sequence (Fryer, 1983; Dorn and Hoffmann, 1983; Olesen, 1999) and in the sequence of the expression of genes involved in the subdivision of the limbs such as annulin (Bastiani *et al.*, 1992) and DSS-8 (Norbeck and Denburg, 1991). All these data suggest an intercalary limb differentiation as has been proposed for *Drosophila* (Goto and Hayashi, 1999) as a general feature for arthropod limb patterning during development. The early establishment of a main P/D axis together with an intercalary differentiation and a similar set of genes (Abzhanov and Kaufman, 2000) allows or even supports the manifold evolution of arthropod limb morphology and function which makes this group so successful.

Fig. 7. Trunk limb homologies between *Cyclestheria hislopi* (left side) and *Leptodora kindtii* (right side). Upper part of figure shows embryos, lower part shows adults. The expression domains of Dll are indicated by brown dots. The six ventral portions of the embryonic limb bud in *Cyclestheria* develop into the median endites and the endopod in the limb of the adult. In *Leptodora*, the five ventral portions of the early limb bud develop into true segments in the adult. This suggests homology between the median trunk limb endites of *Cyclestheria* (and thereby other 'larger branchiopods') and the segments of the raptorial trunk limbs of *Leptodora*. The coloured lines indicate presumed homologous limb parts between *Cyclestheria* and *Leptodora*. The dotted line with a '?' indicates the inclusion of the reduced exopod in the tip of the limb of *Leptodora* caused by the suggested suppression of the bifurcation of the early distal tip of the limb anlage. See text for details.



Materials and Methods

The material of *Cyclestheria hislopi* (Baird, 1859) was collected in Colombia in 1994 (see Olesen *et al.*, 1997) and in Northern Territory, Australia in 1999. *Leptodora kindtii* (Focke, 1844) was collected in 1998 and 1999 in the Tegeler See, Berlin. The material for scanning electron microscopy (SEM) was fixed in glutaraldehyde, formaldehyde or Bouin's Fluid, dehydrated and critical point dried following standard procedures. The other material was fixed in PEM-FA (0.1 PIPES (pH 6.95), 2.0 mM EGTA, 0.1 mM MgSO₄, 3.7% formaldehyde) and stored in methanol. The immunostaining and fluorescent staining followed Scholtz *et al.* (1998). The staging of the embryos of *C. hislopi* followed the guidelines provided by Olesen (1999). The embryos of *L. kindtii* were grouped into 4 appropriate stage categories based on their degree of development.

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