

THE DEVELOPMENT OF PHYLLOPODOUS LIMBS IN LEPTOSTRACA AND BRANCHIOPODA

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ABSTRACT

We present a comparison of the development of trunk limbs in *Nebalia* sp. (Leptostraca) and *Limnadopsis parvispinus* (Branchiopoda). The overall correspondence in specific developmental steps and morphology, e.g., size and orientation of limb anlagen, plus the resulting interpretation of adult limb part homologies lead to the suggestion that phyllopodous limbs in Leptostraca and Branchiopoda are homologous. In addition, our data allow the conclusion that the branchiopod limb forms a three-lobed rather than an undivided endopod. During early development of *Nebalia* sp. the pleopods form a transitory, putative, and vestigial epipod. The presence of this epipod on the pleopod lends support to the idea that the tagmatization of the malacostracan trunk into thorax and pleon is the result of a secondary subdivision of an ancestral crustacean thoracic region.

KEY WORDS: Branchiopoda, developmental biology, evolution, limb patterning, Malacostraca

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INTRODUCTION

Comparative zoologists have discussed for over a hundred years the question of whether the phyllopodous limbs of Branchiopoda and Leptostraca are homologous. Researchers such as Lauterbach (1975), Schram (1986), Hessler (1992), or Ax (1999) favour such a homology. Nevertheless, these authors come to different conclusions concerning the relationships between Branchiopoda and Leptostraca, or Malacostraca as a whole. In contrast to this, Walossek (1993) and Martin and Christiansen (1995) stressed the differences between the limbs of Branchiopoda and Leptostraca and considered convergence of the phyllopodous limb types. These morphological studies were corroborated by a phylogenetic analysis based on molecular data by Spears and Abele (1999), who suggested that the phyllopodous limbs have evolved convergently in the lineages leading to Branchiopoda and Malacostraca. Most comparative morphological analyses discuss Leptostraca as sister group to Eumalacostraca within the Malacostraca (Grobbs, 1892; Hansen, 1893; Siewing, 1956; Dahl, 1987; Richter and Scholtz, 2001), although Schram (1986) finds his Phyllopoda, which includes Leptostraca, Branchiopoda and Cephalocarida, the sister group of Maxillopoda. However, this does not lead to a solution of the problem. Because the phyllopodous limb might be either plesiomorphic for Malacostraca, or an apomorphic character in the stem lineage of Leptostraca; phylogeny alone cannot resolve the morphological relationships of limbs simply described as phyllopodous. While the parts of the phyllopodous limb in Leptostraca are well characterised, there is no general agreement about the different parts of the phyllopodous limbs of Branchiopoda (Borradaile, 1926; Walossek, 1993; Martin and Christiansen, 1995; Ferrari and Grygier, 2003; Waloszek, 2003; Boxshall, 2004; Olesen, 2004, 2007). Furthermore, the limbs of cephalocaridans are

sometimes considered to be phyllopodous (Spears and Abele, 1999).

Recent studies on Branchiopoda (Benesch, 1969; Olesen, 1999a, 2007; Eder, 2002; Møller et al., 2003, 2004; Olesen and Grygier, 2003; Olesen et al., 2003) and with special focus on limbs (Williams and Müller, 1996; Olesen et al., 2001; Ferrari and Grygier, 2003; Williams, 1998, 2004) and Leptostraca (Manton, 1934; Williams, 1998; Olesen and Walossek, 2000) have improved our knowledge of developmental patterns in these taxa. Martin and Christiansen (1995) carried out detailed comparative analyses of the phyllopodous limbs on adult limbs with special focus on the setae.

In this article we compare the pattern of early limb development in representatives of Malacostraca, *Nebalia* sp., and Branchiopoda, *Limnadopsis parvispinus* (Henry, 1924). Our goal is to find attributes of these limbs that will clarify the question of phyllopodous limb homology. In addition, we propose a new interpretation of limb parts in branchiopods and add new evidence to a scenario explaining the malacostracan trunk tagmatization as a secondary subdivision into thorax and pleon.

MATERIAL AND METHODS

Limnadopsis parvispinus

The Australian endemic species *Limnadopsis parvispinus* (Branchiopoda: Spinicaudata) occurs in temporary pools. Resting eggs were collected 1999 by S. Richter from dried mud in northwest New South Wales ('Carters Swamp') (20° 26' S; 144° 59' E). Discrimination between different resting egg types based on shell morphology was necessary because of a co-occurrence of several Limnadiidae in the Paroo area (Timms and Richter, 2002). Resting eggs were allowed to develop in our laboratory in Berlin in freshwater tanks (Petri dishes) with aeration at room temperature. Nauplii and juveniles from resting eggs were fed with *Artemia*-food (®Liquizell). Later on they were fixed in Bouin's fluid (Romeis, 1989) and transferred to ethanol.

Nebalia sp.

The species of the Nebaliidae (Malacostraca: Leptostraca) occur in the littoral and sublittoral zone. Specimens of *Nebalia* sp. were collected in July 2001 by G. Scholtz in the littoral of North Bay on San Juan Island (Washington, USA). Eggs and juveniles were taken from the brood pouches of the mothers, separated, fixed in PEM-FA and transferred to methanol.

Immunocytochemistry

The embryos of *Nebalia* were washed several times in PBS and PBT (PBS + 0,1% BSA + 0,1% Triton) and twice in PBT+N (5% normal goat serum). Afterwards the embryos were incubated overnight at 4°C in PBT+N. The antibody staining followed, using a polyclonal anti-*Distal-less* (*Dll*) primary antibody (1µl/ml) (see Panganiban et al., 1995) plus goat anti-rabbit peroxidase conjugated secondary antibody (1/400) both with overnight incubation at 4°C and followed by several rounds washing in PBS. The colour reaction was developed with DAB (Diaminobenzidine) (for details see Olesen et al., 2001). Embryos were mounted in Glycerol.

SEM

The naupliar and postnaupliar stages of *Limnadopsis parvispinus* and the embryonic and postembryonic stages of *Nebalia* sp. were transferred to 100% ethanol, critical-point-dried (BAL TEC CPD 030) and sputter coated with gold (BAL TEC SCD 005) for SEM examination (LEO 1430).

Light Microscopy and Photography

Light microscopy was done with a Zeiss Axiophot and photography via digital camera (Nikon D1) mounted on a Zeiss Axiophot.

Figure 2A is a summary of a stack of 14 photographs (Fig. 3E of 19 photographs) to enhance the range of the picture elements that are in focus. The calculation was done with Helicon Focus (HeliconSoft).

Stages

The general aspects of the development of *Limnadopsis parvispinus* have been described in detail by Pabst and Richter (2004). In the following, we focus on the development of the trunk limbs including new observations on the differentiation of limb parts. Six larval stages are described in *Limnadopsis parvispinus*. They are defined by distinct morphological characters (see Pabst and Richter, 2004).

For *Nebalia* sp. we applied the embryonic stages (*A* to *I*) as described by Manton (1934) for the related European species *Nebalia bipes* (Fabricius, 1780) (Olesen, 1999b). As in *Limnadopsis parvispinus*, stages of *Nebalia* sp. are also defined via specific external morphological characters.

Abbreviations used in Text and Figures

A-E	lobes of trunk limb anlagen
a	anterior
a1	antennule
a2	antenna
b1-b5	primary bud 1-5
br	brain
ca	carapace
cp	caudal papilla
cr	caudal rami
cu	embryonic cuticle
d	dorsal
e1	enditic lobe 1/proximal endite
en	endopod
ep	epipod
(ep)	epipod-like structure
ex	exopod
la	labrum
md	mandible
mx1	maxillule
mx2	maxilla
ol	optic lobes
p	posterior
pl1-pl4	pleonic limbs 1-4
ro	rostrum
te	telson

th1-th8	thoracic limbs 1-8
tl1-tl8	trunk limbs 1-8
v	ventral

RESULTS

Limb Development in *Nebalia* sp.

The anlagen of the thoracic limbs one and two form transverse buds in stage *A* of *Nebalia* sp. development, which is characterized by the ventrally folded caudal papilla (Fig. 1A). During stages *D-E* eleven trunk limb anlagen (the prospective thoracopods 1-8 and the prospective pleopods 1-3) are present as relatively large transverse buds with the tips pointing laterally (Figs. 1B, 2B, C). The more anterior buds show a slight partition into two lobes, which represent the endopod- and the exopod of the differentiated limbs (Fig. 2E). These two lobes can be recognised up to the limb anlage of the last thoracic segment. A further differentiation in these limb anlagen is not recognizable. The limb anlagen of the thoracic segments one and two are larger than the other limbs. They are still transversally oriented and the two distal lobes point laterally. The prospective endopod is in a ventral and the prospective exopod in a dorsal position.

Stages *F-G* show remarkable morphological changes in comparison to earlier stages (Fig. 1C, D, F). The caudal papilla is now posteriorly oriented. The limb anlagen up to the third pleonic segment are present. The tips of all limb anlagen show a subdivision into the two lobes of the endopod and exopod. The thoracic limb anlagen are still oriented transversely but they also point slightly posteriorly (Fig. 1F). All thoracic limb anlagen appear in the medio-lateral body region and occupy the biggest part of the segment (Fig. 1D). The area between the basal parts of the limb anlagen within a segment is occupied by the forming ganglia of the central nervous system. In contrast to this, the pleonic limb anlagen are in a more ventral position and oriented posteriorly (Fig. 1D, F). Accordingly, the lobe of the endopod appears medially and the lobe of the exopod laterally. In stage *I* the thoracic limb anlagen change their orientation to ventral with the tips of the endo- and exopods pointing downwards (Fig. 1E). The prospective endopod and exopod of each limb are similar, but the endopod is slightly longer, and the exopod is slightly wider. The anlage of the epipod is present on the thoracic limbs (Fig. 3B, D). It can be recognized somewhat distal to the exopod and exhibits a rounded tip. The anterior three pleopods are oriented ventroposteriorly (Fig. 1G). The prospective endopod and exopod are of the same length. The pleopods four to six are present as small, undifferentiated buds. Interestingly, the anteriormost three pairs of pleopods show an anlage of a third lobe proximal to the prospective exopod (Figs. 1F, 3A, C). These lobes are distally pointed and appear in the same position as the epipod anlage of the thoracic limbs (Fig. 3B, D). The thoracic limbs of the first postembryonic stage show basically the shape of the adult limbs (Fig. 3E) but with a clear separated endopodal segment distally and a constriction between the prospective coxa and basis (Fig. 3E).

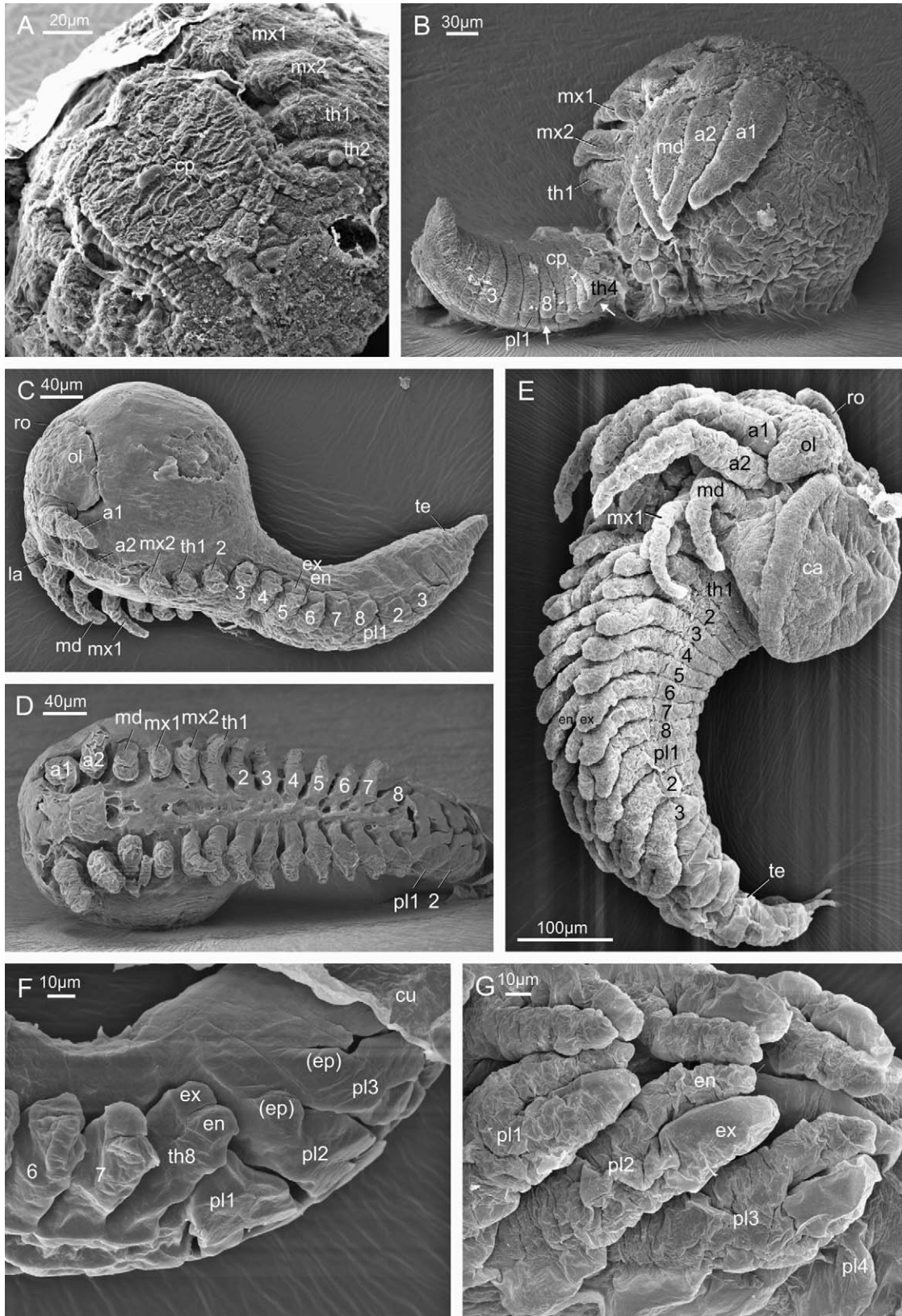


Fig. 1. Limb development of *Nebalia* sp., SEM. A, stage A, posteroventral view of the postnaupliar body region with the caudal papilla and the anlagen of the maxillule up to the thoracic limb 2; B, stages D-E, lateral view of a whole specimen, arrows indicate the partition of the limb anlagen, the caudal papilla is folded back from the remaining body for a better view, anterior is right; C, stage G, lateral view of a whole specimen, anterior is up; D, stage G, ventral view of a whole specimen, anterior is left; E, stage I, lateral view of a whole specimen, anterior is up; F, stage G, lateral view of the left thoracic limb anlage 6 up to the pleonic limb anlage 3, anterior is left; G, stage I ventral view of the pleonic limb anlage 1 to 4, anterior is left.

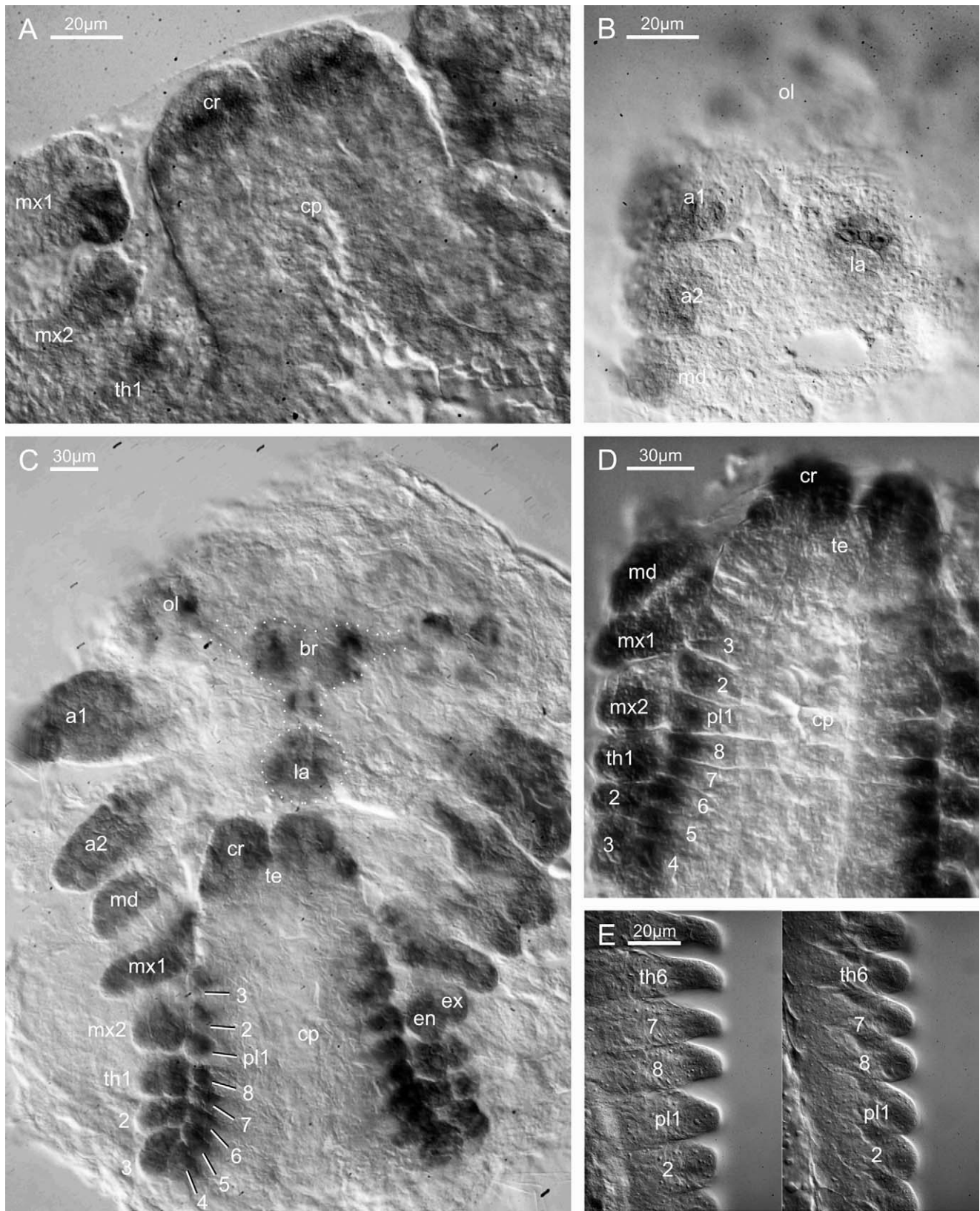


Fig. 2. *Dll* expression during limb development of *Nebalia* sp., light microscopy. *Dll* positive areas with dark coloration, A construct with Helicon Focus (HeliconSoft). A, stage A, ventral view of the caudal papilla, anterior is up; B, stage A, ventral view of the naupliar region, anterior is up; C, stage D, ventral view of the whole specimen, non embryonic tissue was removed, the caudal papilla is situated ventrally, anterior is up; D, stage D, ventral view of the caudal papilla, anterior is up; E, stage E, ventral view of the left side of the caudal papilla, thoracic limb anlagen 5 up to 8 and pleonic limb anlagen 1 to 3, left image is focused on the prospective endopod, right image is focused on the prospective exopod, anterior is up.

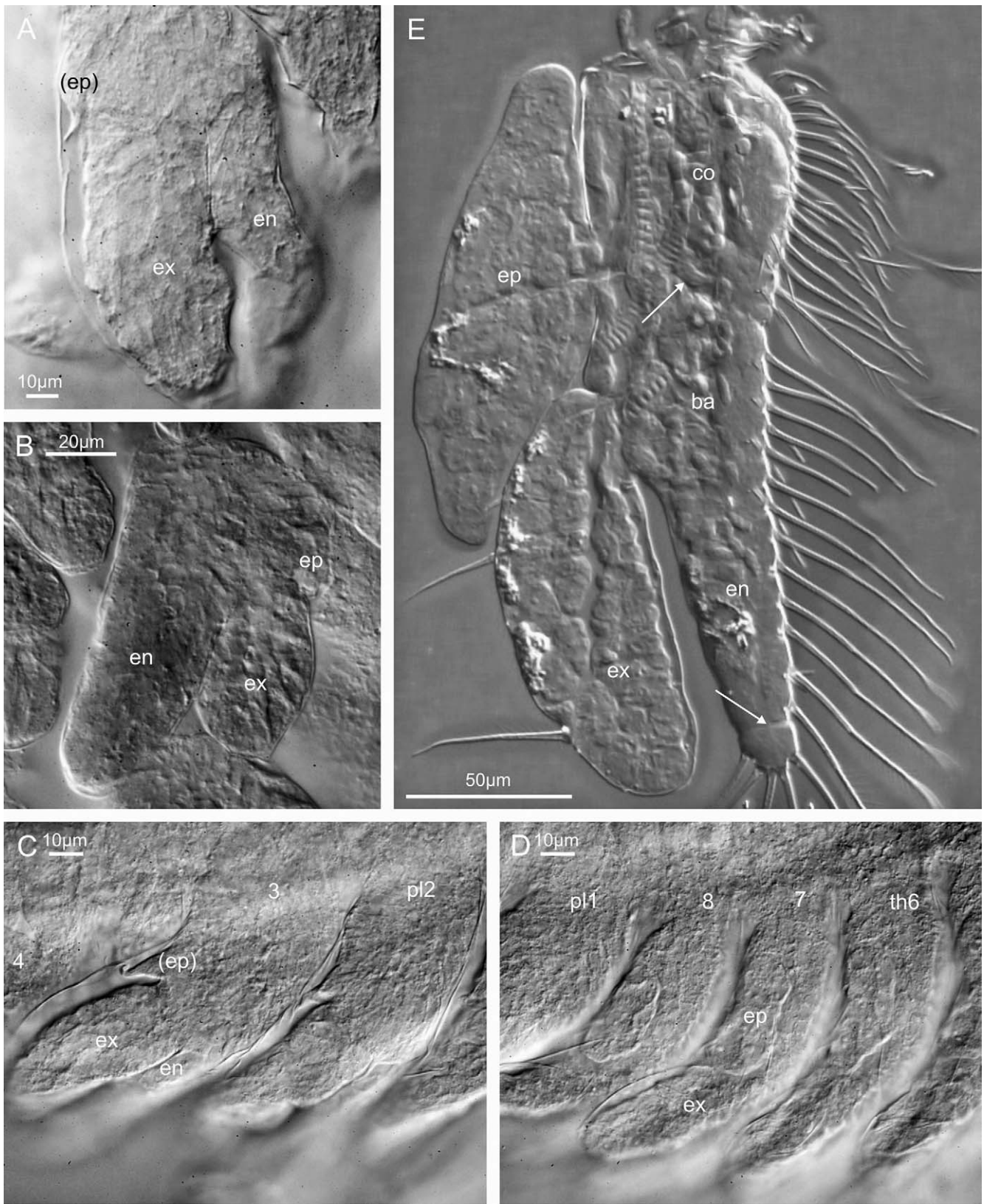


Fig. 3. Epipod development in *Nebalia* sp., light microscopy. C construct with Helicon Focus (HeliconSoft). A, frontal view of a right pleopod anlage, dorsal is up; B, frontal view of a thoracopod anlage, dorsal is up; C, stage I, lateral view of the pleonic limb anlagen 2 to 4, anterior is right, dorsal is up; D, stage I, lateral view of the thoracic limb anlagen 6 to 8 and pleonic limb anlage 1 anterior is right, dorsal is up; E, frontal view of the left thoracic limb 7 of a hatched juvenile specimen, segmental constrictions are indicated by arrows.

The *Dll* Expression Pattern During Limb Development in *Nebalia* sp.

The early morphogenesis of limbs is accompanied with a clear expression of *Dll*. Stage A of *Nebalia* sp. exhibits groups of *Dll* positive cells in the distal portion of the lobe-like buds of the antennules to the maxillules (Fig. 2A, B). The transverse buds that indicate the prospective biramous maxillae and the first thoracic pair of limbs are also each marked by a cluster of *Dll* positive cells (Fig. 2A). Additional areas with *Dll* expression are found in the brain, in the forming labrum (2B), and in the two terminal lobes (caudal rami) of the forming caudal papilla (Fig. 2A). All these *Dll*-positive domains can also be found in further developed embryos of stage D (Fig. 2C, D). In stage D, the size of the limb anlagen of the antennules to the maxillules has increased, and the anlagen of the maxillae and the thoracic limbs exhibit a partition into the two lobes of the endopod and exopod both of which express *Dll*. The number of limb anlagen and hence the number of *Dll* positive areas is increased up to the third anlage of pleopods (Fig. 2C, D). All these limb anlagen with *Dll* expression are in a lateral-most position on the caudal papilla (Fig. 2C, D). The two caudal rami of the caudal papilla are larger and the whole area exhibits a strong *Dll* expression (Fig. 2C, D).

Limb Development in *Limnadopsis parvispinus*

The first two or three paired trunk limb anlagen of *Limnadopsis parvispinus* larvae are recognizable as undifferentiated buds in ventrolateral orientation on each side of the body in stage III (Fig. 4A). During stage IV the number of trunk limb anlagen increases to five (Fig. 4B). These are still not subdivided into the various limb parts characteristic for Branchiopoda. This changes during stage V, in which the anterior limb anlagen one to five are differentiated to varying degrees (Fig. 4C, D). These differentiated limb anlagen comprise the biggest part of the larval segments (Fig. 4C, D, E). They reach far medially leaving a median space that is occupied by the forming ganglia of the central nervous system (see Harzsch, 2001; Olesen et al., 2001, 2003). Two additional pairs of limb anlagen are present as undifferentiated buds (Fig. 4C arrows). The anterior four limb anlagen are subdivided into eight lobes each (Fig. 4D, t14). The lobes two and three and the lobes four to six have a developmental origin from one primary bud each (Fig. 4D, t13, t14). This means that the early trunk limb anlage is originally subdivided into five primary buds (Fig. 4D, t15). These comprise primary bud one—first enditic lobe; primary bud two—enditic lobes two and three; primary bud three—enditic lobes four to five and the so called endopod; primary bud four—exopod anlage and primary bud five—epipod anlage. The first enditic lobe is the largest.

The fifth pair of trunk limbs has no epipod anlage at this stage. The entire array of limb anlagen are horizontally (mediolaterally) oriented (Fig. 4C) and the distalmost parts of the limbs are directed laterally (Fig. 4D). In stage VI five to six limb anlagen show a differentiation into eight lobes. Again, the anlage of the epipod is not present in the posteriormost of the differentiated limbs. In addition, one or two undifferentiated limb anlagen are formed (Fig. 4E).

the postlarval stage I, the anterior four trunk limbs are almost completely differentiated and used for swimming (Fig. 4E). With further development the tips of the limbs represented by the endo- and exopods occupy a ventral position with the tips pointing downwards. Two more pairs of trunk limbs are differentiated to varying degrees, and two more limb pairs are present as undifferentiated buds.

DISCUSSION

Evidence for Pleonic Epipods and the Origin of Pleopods

Most of our data in *Nebalia* sp. confirm the general pattern of leptostracan (limb) development (Manton, 1934; Olesen and Walossek, 2000; Williams, 2004). Nevertheless, some of our results add new aspects to the discussion of leptostracan limb development and evolution.

During the early limb development of *Nebalia* sp. the anlagen of thoracopods and of the first three pleopods are very much alike and there is no distinct gap in the level of differentiation between the thoracic and the pleonic limbs as is observed in other malacostracans (see Scholtz, 1992, 1995; Ungerer and Wolff, 2005; Alwes and Scholtz, 2006). This is an indication of how serially homologous limbs were patterned in the stem lineage of Malacostraca before limbs became differentiated as thoracopods and pleopods (Olesen and Walossek, 2000). In adult Leptostraca, no pleopodal structure can be found comparable to the epipod of the thoracopods. However, late embryonic stages of *Nebalia* sp. show a lobe-like structure on the anlagen of the pleopods one to three. This lobe is localized proximal to the prospective exopod in a corresponding position to that of the epipod anlage of the thoracopods. In addition, it exhibits a similar shape to thoracic epipod anlagen apart from the rounded tip of the latter. Furthermore, this lobelike structure appears relatively late during limb differentiation, as seems typical for epipods (Wolff and Scholtz, 2008). Accordingly, we suggest homology of the pleopod lobes to the thoracic epipod anlagen. This is the first evidence for pleopodal epipods in Malacostraca, if the gills on the pleopods of stomatopods are not interpreted as such. The further development of these pleonic epipodal lobes in *Nebalia* is not clear. Apparently they are reduced during the course of development. On the other hand, the epipodal lobes could form the large setae that are found in a corresponding position in adults. The interpretation of setae as vestiges of epipods on the outer coxal margin in copepod maxillae and ostracode maxillulae (Boxshall, 2004) supports this view. Interestingly, the Cambrian fossil *Yicaris dianensis* (Zhang et al., 2007), which is interpreted as crown-group crustacean (Zhang et al., 2007) also exhibits epipods, which apparently are derived from a bulb-like structure with a terminal spine.

Olesen and Walossek (2000) discuss a possible fusion of coxa and basis in the pleopods one to three of *Nebalia brucei* (Olesen, 1999b). Moreover, Ungerer and Wolff (2005) interpret a constriction in the proximal part of pleopods in early developmental stages of the amphipod *Orchestia cavimana* (Heller, 1865) as indication of a coxa and basis. Traditionally, the protopod of pleopods is viewed as the result of the fusion of the coxa and basis (Thiele, 1905; Borradaile, 1926). A different view is expressed by

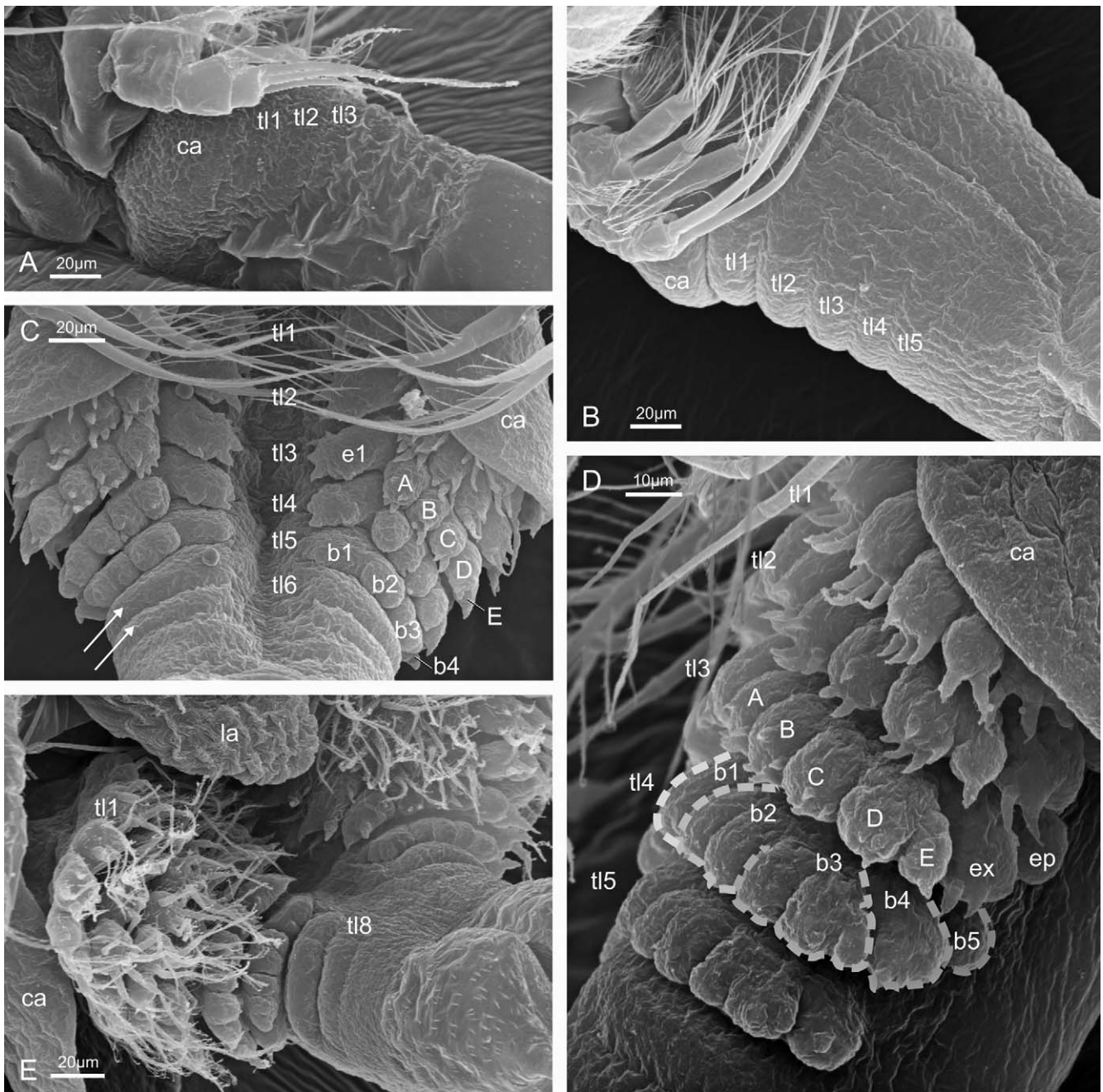


Fig. 4. Limb development of *Limnadopsis parvispinus*, SEM. A, C-E modified after Pabst and Richter (2004). A, stage III, lateral view of the right anlagen of the carapace and trunk limbs 1 to 3, anterior is up and to left; B, stage IV, ventral view of the trunk area with the anlagen of the carapace and trunk limbs 1 up to 5, anterior is up and to left; C, stage V, ventral view of the trunk region with the anlagen of trunk limbs 1 to 6, anterior is up and to right; D, stage V, lateral view of the left trunk region with the anlagen of trunk limbs 1 to 6, the primary buds 1 to 3 and the buds 4 (exopod) and 5 (epipod) are indicated via dotted lines (limb anlage 4), anterior is up and to right; E, postnaupliar stage I, posteroventral view of the trunk area with the trunk limbs 1 to 8, anterior is up and to left.

Walossek (1993), who supposes a plesiomorphically single proximal segment in the pleopods of malacostracans formed exclusively by the basis and without a participating coxa. In contrast to this view, the investigations in *O. cavimana* and *Nebalia* sp. allow for a secondarily unsegmented proximal part.

These data and suggestions provide further support for the view that the entire trunk of malacostracans corresponds to the trunk (thorax) of non-malacostracan crustaceans, and that it is only secondarily subdivided into a “thorax” and a pleon (Lauterbach, 1975; Scholtz, 1995; Walossek and Müller, 1997; Ax, 1999; Abzhanov and Kaufman,

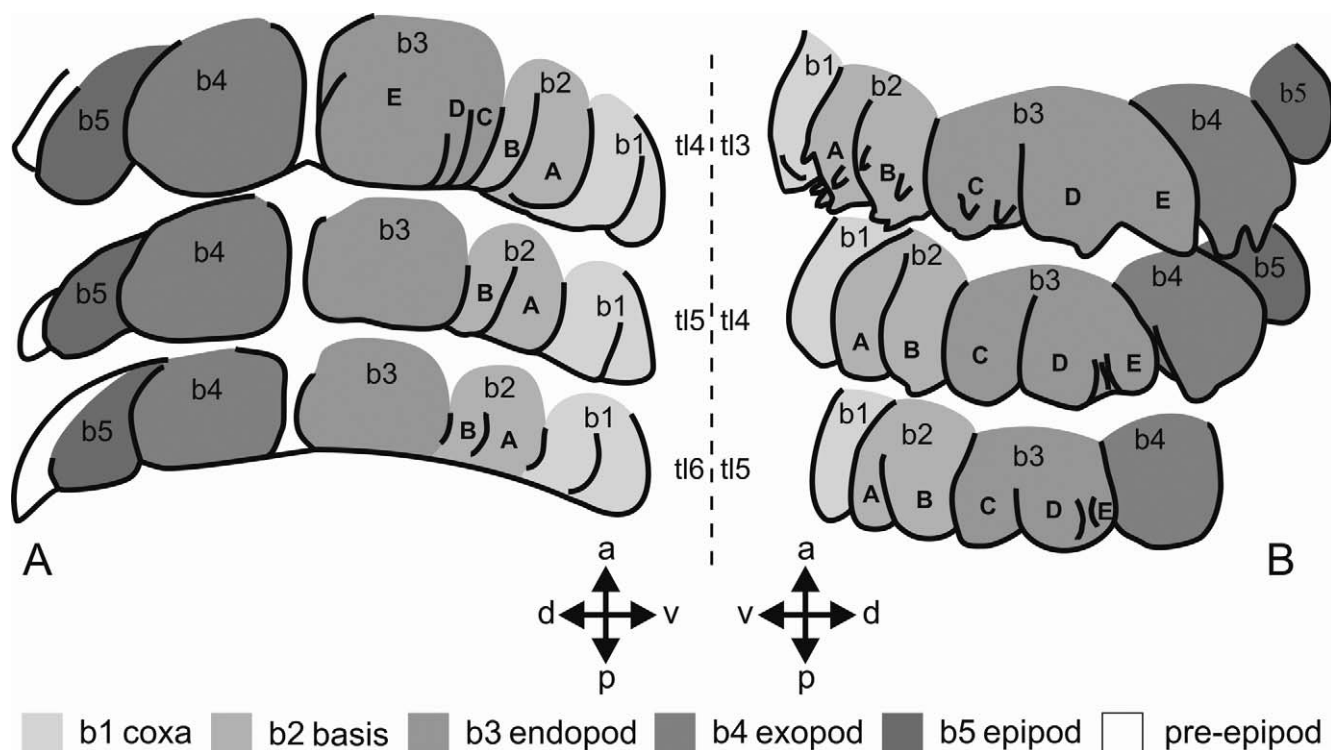


Fig. 5. Schematic representations of limb anlagen of Anostraca and Phyllopora. A, Anostraca: *Eubranchipus grubii* (modified from Møller et al., 2004), scheme of stage with 5 to 7 well developed trunk limbs; B, Phyllopora: *Limnadopsis parvispinus* (modified after Pabst and Richter, 2004), scheme of stage V, lateral view of left trunk limb anlagen 3 to 5.

2004; Olesen and Walossek, 2000; Schram and Koene-
mann, 2004).

The Development of the Phyllopodous Limbs of Branchiopoda

The appendages of the various taxa within the Branchiopoda exhibit a similar composition (Walossek, 1993; Olesen, 2004, 2007). Endites one to five often are assumed to be homologous structures (Walossek, 1993; Boxshall, 2004; Williams, 2007; Olesen, 2007). The putative endopod is situated next distal to the fifth endite. It is present as terminal endite-like structure and is often interpreted as secondarily unsegmented (Waloszek, 2003; Olesen, 2004, 2007; Boxshall, 2004). However, see Martin and Christiansen (1995) on *Leptestheria kawachiensis* (Uéno, 1927) and Heath (1924), Borradaile (1926), and Benesch (1969) on Anostraca for different opinions.

The argument in favour of an undivided small endopod in Branchiopoda is mainly based on the limbs of the Upper Cambrian fossil species *Rehbachella kinnekullensis* Müller, 1983 (Walossek, 1993). *Rehbachella* shows a segmented endopod and the joint between this segmented endopod and the limb basis is interpreted as being homologous to the cuticular line separating the distal-most endite-like structure and the more basal elements of the limbs of recent branchiopods (see Olesen, 2007). However, a Cambrian fossil would not necessarily exhibit the plesiomorphic character state. Because the limb anlagen and differentiation of *Rehbachella* are very different from the common branchiopod pattern, it is far from clear whether *Rehba-*

chiella shows the original condition for branchiopod limbs (see also Schram and Koene-
mann, 2001). *Rehbachella* may have already lost the clear segmentation of the proximal endite bearing part of the endopod, i.e., even if the cuticular line is homologous as Olesen (2007) suggests, it might not represent the boundary between the basis and the endopod.

In our study of the limb development of *Limnadopsis parvispinus*, we found a distinct temporal and spatial hierarchy in the patterning of the early limb bud into distinct lobes. One can distinguish five primary buds forming the early limb anlage. Four of these primary buds are differentiated into the larval lobes that in the end form the enditic lobes, the exopod and the endopod. The epipods appear somewhat later at the dorsal margin of the limb anlagen (Fig. 5). This pattern of a stepwise differentiation of the initial limb anlagen allows some conclusions concerning morphological and morphogenetic entities of branchiopod phyllopodous limbs which might contribute to the highly controversial discussion about homologies of the individual limb parts between the large branchiopod groups and with respect to crustacean limbs in general. The resulting view is somewhat different from the almost generally held view on how to interpret branchiopod limbs (see Olesen, 2007; Williams, 2007). Based on this we apply a different terminology to the forming lobes.

Evidence for a Three-segmented Endopod.—Lobe E in our terminology is the smallest lobe in all limb anlagen of stage V (Figs. 4D, 5B). In the commonly held hypothesis of the composition of branchiopod limbs, lobe E is the anlage of

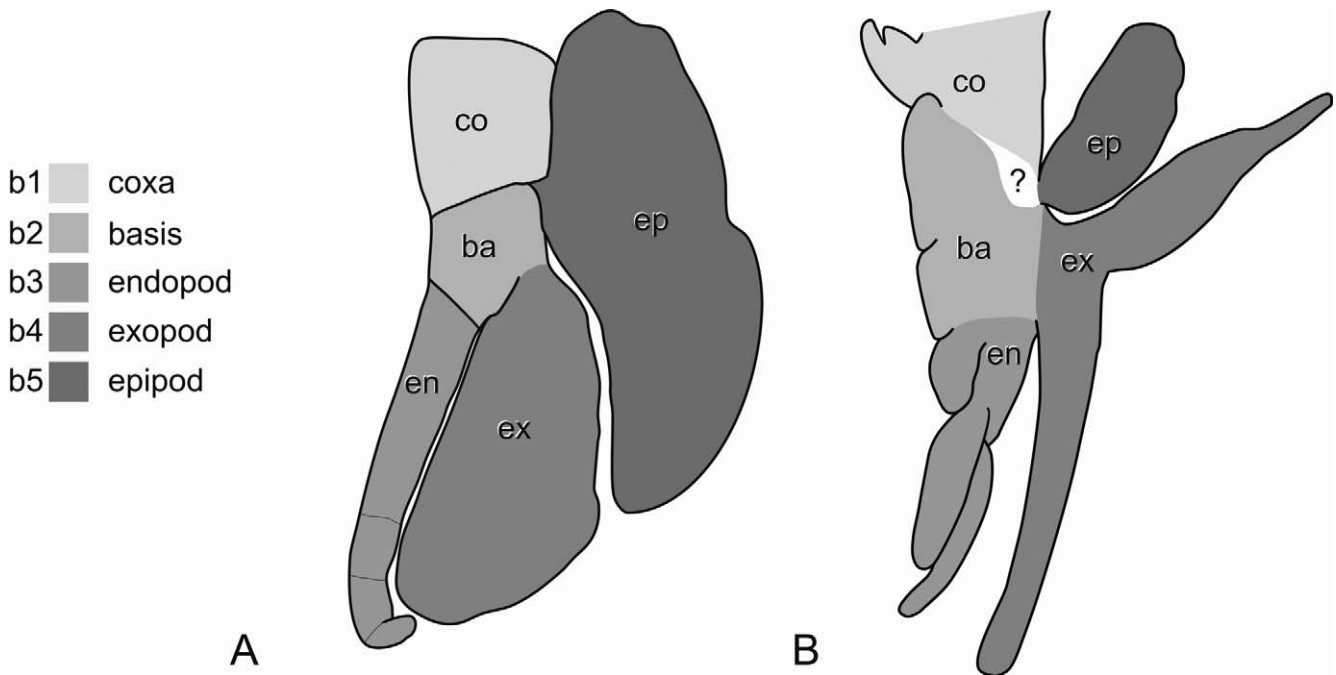


Fig. 6. Schematic representations of phyllopodous limbs. A, thoracic limb of *Nebalia* sp.; B, trunk limb of *Limnadopsis parvispinus*. In *Nebalia* the epipod is connected to coxa and basis (see Fig. 3E). The question mark in the *Limnadopsis* limb indicates some insecurity concerning the boundary between coxa and basis, and the attachment site of the epipod. b1 to b5 refer to the primary buds in the forming limbs of *Limnadopsis*.

the secondarily unsegmented endopod, but the development of the lobes C, D and E does not match this hypothesis (Figs. 4D, 5B). All these lobes have their origin from one primary bud (bud 3) (Figs. 4C, D, 5B). This suggests that the primary bud 3 as a whole gives rise to the endopod and that the endopod is thus tripartite, i.e., it is formed by three enditic lobes (Fig. 6A). A tripartite endopod has also been described for *Leptestheria kawachiensis*, another spinicaudate species (at least for females, males possess even four endopodal parts) (Ferrari and Grygier, 2003). The conclusion of Ferrari and Grygier (2003) is based on the observation that the limb is patterned at two locations, adjacent to the body wall and in a restricted area distally. The landmark of an arthrodial membrane on more posterior limbs and on developmentally early anterior limbs allowed a detailed comparison of branchiopod and copepod limbs (Ferrari and Grygier, 2003). Further evidence for tripartite endopods results from the occurrence of clearly three-segmented endopods in Onychopoda among cladocerans (Hansen, 1925; Olesen et al., 2003; Olesen, 2004). However, the latter example raises the question whether the three enditic lobes in phyllopodous limbs correspond to segments in stenopodous limbs. A strong indication for this is the clear evidence that the segments of the stenopodous limbs of the raptorial water flea *Leptodora kindtii* (Focke, 1844) are evolutionarily derived from the lobes of phyllopodia seen in other cladocerans (see Olesen et al., 2001).

From this it follows that a secondarily “unsegmented” endopod, which has its origin only in lobe E in phyllopodous limbs of Branchiopoda, is at least very unlikely. On the contrary, there is much evidence in favour

of the view that a three-partite endopod, either lobed or segmented, is the general pattern for Branchiopoda as has been already proposed by Hansen (1925).

A New Interpretation of the Endites in Branchiopoda.—The development of the anostracan *Eubranchipus grubii* (Dybowski, 1860) has been investigated by Møller et al. (2004). Eleven lobes occur in the forming limbs of this species. According to these authors, these lobes comprise the six median lobes forming the endites, the endopod lobe, the exopod, and three exiteanlagen. The endites (proximal endite and the endite 2), which are in the most median position, fuse during ontogeny to form the proximal largest enditic lobe in adult trunk limbs, as is also discussed by Cannon (1933), Lowndes (1933), Fryer (1983) and Williams (2007) for other anostracans. The general pattern of lobe formation in Phyllopoda is slightly different in that only five putative enditic lobes are formed median to the putative endopod (see Olesen, 2004, 2007). Møller et al. (2004) suggest a correspondence of the lobes one and two to the lobes one and two in “conchostracans”. This means that lobe A of primary bud 2 of *Limnadopsis parvispinus* in our terminology corresponds to lobe two of Anostraca. It also implies that, although in adults of most Anostraca and in Phyllopoda there are five inner lobes, the homology of these lobes is not resolved. It is furthermore unclear, which lobe has been lost or fused in the lineage leading to Phyllopoda. Olesen (2007) just states that five median lobes are an apomorphy for Phyllopoda.

In the following we present an alternative view trying to resolve this issue. Based on the recognition of primary buds that develop into a defined number of secondary lobes, we

suggest that the lobes A and B in our terminology are subunits of one broad structure, which corresponds to the primary bud 2 (Fig. 5). The innermost primary bud marks the most proximal part of the limb and might thus correspond to the two innermost lobes in the anostracans (Fig. 5). Apart from some taxa, all Anostraca show a fusion of the two most proximal enditic lobes during development. The median endite A of *Limnadopsis parvispinus* is represented by bud 1 as only one lobe. It shows no distinct sign of fusion with another lobe. This seems true for other phyllopodans as well (see Møller et al., 2004; Olesen, 2007). Thus, in contrast to the situation in Anostraca, we cannot say whether the situation in Phyllopoda is due to a fusion (convergent to Anostraca) or to loss of one of the two innermost buds found in Anostraca. The finding of Ferrari and Grygier (2003), who describe an antennate lobe associated with enditic lobe 1, might indicate the originally two-part nature of the median lobe in Phyllopoda. In *Limnadopsis parvispinus*, three primary buds give rise to the adult median part of the limb. This is a general pattern of Phyllopoda (see Olesen, 2004, fig. 9a, 10a, 2007, fig. 11c). According to our view, the primary bud 4 develops into the exopod. In summary, we suggest that the lobes of the anostracans and phyllopodans can be aligned and homologised one by one with the exception of the two innermost endites of Anostraca, which correspond just to one endite in Phyllopoda (Fig. 5).

Based on the proximal patterning of a limb adjacent to the body wall and a proximal arthrodistal membrane, Ferrari and Grygier (2003) proposed the existence of a basis, a coxa, and a precoxa for the two proximal parts of the phyllopodous limb in Branchiopoda, in agreement with Hansen (1925). However, the parts of the limb that comprise the basis and the coxa according to the hypothesis of Ferrari and Grygier (2003) originate in one primary bud in *Limnadopsis parvispinus* and the other Phyllopoda.

Our interpretation of the phyllopodous limb of Branchiopoda agrees with the hypothetical ancestral crustacean maxillula as proposed by Boxshall (1997). This view comprises a coxa and a basis with two subunits each, a segmented endopod and an unsegmented exopod. The only difference is the lack of an epipod in the ancestral maxillula.

A Comparison of the Phyllopodous Limbs of *Nebalia* and *Limnadopsis*

The limb anlagen of *Nebalia* sp. and *Limnadopsis parvispinus* show a number of similarities in their development. In both species, limb anlagen appear first as transverse, lateroventral buds. In both taxa, the anlagen of the limbs comprise the largest part of the segments and reach far medially. The distance between the ventralmost (basal) part of the paired limb anlagen within a segment corresponds to the area of the forming ganglia of the ventral central nervous system. The first differentiation in the limb anlagen is a partition into two distal lobes in *Nebalia* sp. The lobes are the prospective endopod (ventral) and the prospective exopod (dorsal). This somewhat different to *Limnadopsis parvispinus* where a larger number of lobes are present at the beginning of limb bud differentiation. Nevertheless, the most lateral lobes represent the pro-

spective endopod (ventral) and the prospective exopod (dorsal). This is also reflected by the expression of the *Distal-less* gene, which in branchiopod and leptostracan representatives is found first in the tips of the prospective exopod and endopod (Panganiban et al., 1995; Williams, 1998, 2004; Olesen et al., 2001; this study). In *Nebalia* sp. and *Limnadopsis parvispinus* the limbs that are part of the adult filter feeding apparatus change their position from horizontal orientation to a ventral orientation during further development. This results from the elongation of the lateral parts of the limb anlagen. Williams (2007) described a rotating of the proximal/distal axis from lateral to distal as similar way of development in Branchiopoda and Leptostraca.

In contrast, the trunk limbs of other malacostracans, namely eumalacostracans, and those of other crustaceans such as Remipedia are formed differently. In most cases that have been studied the limb buds appear as relatively small areas at the ventral side of the embryo or larvae with the tips pointing ventrally from the onset (Scholtz, 1992; Hejnol and Scholtz, 2004; Alwes and Scholtz, 2006; Koenemann et al., 2007).

However, it has to be stressed that the kind of large transverse limb buds with laterally pointing distal regions does not necessarily lead to phyllopodous limbs. The contrary is exemplified by the limbs of the cladoceran *Leptodora* and by the appendages of the pleon of *Nebalia* (Olesen and Walossek, 2000; Olesen et al., 2001; this study). *Leptodora* shows the same initial limb buds and lobe differentiation as other cladocerans and branchiopods. Nevertheless, the resulting limbs are segmented stenopodous legs and not phyllopodia (Olesen et al., 2001). The same is partly true for the pleopods of *Nebalia*, which show serially homologous early limb buds with those of the thoracic region. Only later in development they adopt a different fate. However, the stenopodous limbs in *Leptodora* are clearly secondarily evolved from phyllopodous limbs (Olesen et al., 2001), and this might also be the case for the pleopods in leptostracans (Lauterbach, 1975).

Thus, the overall similarities in the general developmental pattern of limbs in *Nebalia* sp. and *Limnadopsis parvispinus* lead us nonetheless to conclude that phyllopodous limbs in Leptostraca and Branchiopoda appear to be homologous structures (Fig. 6).

The view of homology of branchiopod and malacostracan phyllopodia also is supported by fossil evidence. The tagmosis and general appearance the Silurian *Cinerocaris magnifica* (Briggs et al., 2004) suggests that this species is a malacostracan with probable close affinities to recent Leptostraca (Briggs et al., 2004). Interestingly enough, this species possesses phyllopodous thoracic limbs, which represent some kind of intermediate condition between branchiopod and malacostracan phyllopodia. The overall shape resembles modern leptostracan thoracic limbs (apart from the unusually high number of putative exopod and epipod flaps, see Briggs et al., 2004), but the limb stem and the inner branch are equipped with six or seven endites as in the branchiopods.

Walossek (1993) rightly points to the differences in the filter mechanisms between Leptostraca and Branchiopoda.

However, this does not necessarily exclude homology, since a hypothesis of homology should be based on the correspondence of similar parts between two structures and is not falsified by differences (Scholtz, 2005).

Whether the homology of phyllopodous limbs propose here indicates a sister group relationship for Malacostraca and Branchiopoda (see Lauterbach, 1975; Richter, 2002) or other close affinities (Schram, 1986; Ax, 1999) must remain open at present.

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REFERENCES

- Abzhanov, A., and A. Kaufmann. 2004. *Hox* genes and tagmatization of the higher Crustacea (Malacostraca), pp. 43–74. In: G. Scholtz (ed.), *Evolutionary Developmental Biology of Crustacea*. Crustacean Issues 15. A. A. Balkema, Lisse.
- Alwes, F., and G. Scholtz. 2006. Stages and other aspects of the embryology of the parthenogenetic Marmorkrebs (Decapoda, Reptantia, Astacida). *Development Genes and Evolution* 216: 169–184.
- Ax, P. 1999. *Das System der Metazoa*. Ein Lehrbuch der phylogenetischen Systematik. Bd. II. Fischer Verlag, Stuttgart. 384 pp.
- Benesch, R. 1969. Zur Ontogenie und Morphologie von *Artemia salina* L. *Zoologische Jahrbücher, Anatomie und Ontogenie der Tiere* 86: 307–458.
- Borradaile, L. A. 1926. Notes upon crustacean limbs. *Annals and Magazine of Natural History* (9th Series) 17: 193–213.
- Boxshall, G. A. 1997. Comparative limb morphology in major crustacean groups: the coxa-basis joint in postmandibular limbs. pp. 155–167. In: R. A. Fortey and R. H. Thomas (eds.), *Arthropod Relationships*. Systematics Association Special Volume Series 55. Chapman and Hall, London. 403 pp.
- . 2004. The evolution of arthropod limbs. *Biological Reviews* 79: 253–300.
- Briggs, D. E. G., M. D. Sutton, D. J. Siveter, and D. J. Siveter. 2004. A new phyllocarid (Crustacea: Malacostraca) from the Silurian fossil-lagerstätte of Herefordshire, UK. *Proceedings of the Royal Society of London B* 271: 131–138.
- Cannon, H. G. 1933. On the feeding mechanism of the Branchiopoda. *Philosophical Transactions of the Royal Society of London B* 222: 267–352.
- Dahl, E. 1987. Malacostraca maltreated - the case of the Phyllocarida. *Journal of Crustacean Biology* 7: 721–726.
- Eder, E. 2002. SEM investigations of the larval development of *Imnadia yeyetta* and *Lepthesteria dahalacensis* (Crustacea: Branchiopoda: Spinicaudata). *Hydrobiologia* 486: 39–47.
- Ferrari, F. D., and J. M. Grygier. 2003. Comparative morphology among trunk limbs of *Caenestheriella gifuensis* and *Leptestheria kawachiensis* (Crustacea: Branchiopoda: Spinicaudata). *Zoological Journal of the Linnean Society* 139: 547–564.
- Fryer, G. 1983. Functional ontogenetic changes in *Branchinecta ferox* (Milne-Edwards) (Crustacea: Anostraca). *Philosophical Transactions of the Royal Society London B* 303: 229–343.
- Grobben, K. 1892. Zur Kenntnis des Stammbaums und des Systems der Crustaceen. *Sitzungsberichte der Mathematisch-Naturwissenschaftlichen Classe der Kaiserlichen Akademie der Wissenschaften Wien* 101: 237–274.
- Hansen, H. J. 1893. Zur Morphologie der Gliedmaßen und Mundtheile bei Crustaceen und Insecten. *Zoologischer Anzeiger* 16: 193–212.
- . 1925. *Studies on Arthropoda II*. On the comparative morphology of the appendages in the Arthropoda. A. Crustacea. *Gyldendalske Boghandel, Kjøbenhavn*. 176 pp.
- Harzsch, S. 2001. Neurogenesis in the crustacean ventral nerve chord: homology of neuronal stem cells in Malacostraca and Branchiopoda?. *Evolution & Development* 3: 154–169.
- Heath, H. 1924. The external development of certain phyllopod. *Journal of Morphology* 38: 453–483.
- Hejnol, A., and G. Scholtz. 2004. Clonal analysis of *Distal-less* and *engrailed* expression patterns during early morphogenesis of uniramous and biramous crustacean limbs. *Development Genes and Evolution* 214: 473–485.
- Hessler, R. R. 1992. Reflections on the phylogenetic position of the Cephalocarida. *Acta Zoologica* 73: 315–316.
- Koenemann, S., F. R. Schram, A. Bloechl, T. M. Iliffe, M. Hoememann, and C. Held. 2007. Post-embryonic development of remipede crustaceans. *Evolution & Development* 9: 117–121.
- Lauterbach, K.-E. 1975. Über die Herkunft der Malacostraca (Crustacea). *Zoologischer Anzeiger* 194: 165–179.
- Lowndes, A. G. 1933. The feeding mechanism of *Chirocephalus diaphanous* Prévost, the fairy shrimp. *Proceedings of the Zoological Society of London* 1933: 1093–1118.
- Manton, S. M. 1934. On the embryology of the Crustacean *Nebalia bipes*. *Philosophical Transactions of the Royal Society London B* 223: 163–238.
- Martin, J. W., and J. C. Christiansen. 1995. A morphological comparison of the phyllopodous thoracic limbs of a leptostracan (*Nebalia* sp.) and a spinicaudate conchostracan (*Leptestheria* sp.), with comments on the use of Phyllopoidea as a taxonomic category. *Canadian Journal of Zoology* 73: 2283–2291.
- Møller, O. S., J. Olesen, and J. T. Høeg. 2003. SEM studies on the early development of *Triops cancriformis* (Bosch) (Crustacea, Branchiopoda, Notostraca). *Acta Zoologica* 84: 267–284.
- , ———, and ———. 2004. On the larval development of *Eubranchipus grubii* (Crustacea, Branchiopoda, Anostraca), with notes on the basal phylogeny of the Branchiopoda. *Zoomorphology* 123: 107–123.
- Müller, K. J. 1983. Crustacea with preserved soft parts from the Upper Cambrian of Sweden. *Lethaia* 16: 93–109.
- Olesen, J. 1999a. Larval and post-larval development of the branchiopod clam shrimp *Cyclestheria hislopi* (Baird, 1859) (Crustacea, Conchostraca, Spinicaudata). *Acta Zoologica* 80: 163–184.
- . 1999b. A new species of *Nebalia* (Crustacea, Leptostraca) from Unguja Island (Zanzibar), Tanzania, East Africa, with a phylogenetic analysis of leptostracan genera. *Journal of Natural History* 33: 1789–1810.
- . 2004. On the ontogeny of the Branchiopoda (Crustacea): contribution of development to phylogeny and classification. pp. 217–269. In: G. Scholtz (ed.), *Evolutionary developmental biology of Crustacea*. Crustacean Issues 15. A. A. Balkema, Lisse. 285 pp.
- . 2007. Monophyly and phylogeny of Branchiopoda, with focus on morphology and homologies of branchiopod phyllopodous limbs. *Journal of Crustacean Biology* 27: 165–183.
- , and M. J. Grygier. 2003. Larval development of Japanese 'conchostracans': part 1, larval development of *Eulimnadia braueriana* (Crustacea, Branchiopoda, Spinicaudata, Limnadiidae) compared to that of other limnadiids. *Acta Zoologica* 84: 41–61.
- , and D. Walossek. 2000. Limb ontogeny and trunk segmentation in *Nebalia* species (Crustacea, Malacostraca, Leptostraca). *Zoomorphology* 120: 47–64.
- , S. Richter, and G. Scholtz. 2001. The evolutionary transformation of phyllopodous to stenopodous limbs in the Branchiopoda (Crustacea) – Is there a common mechanism for early limb development in arthropods?. *International Journal of Development* 45: 869–876.
- , ———, and ———. 2003. On the ontogeny of *Leptodora kindtii* (Crustacea, Branchiopoda, Cladocera), with notes on the phylogeny of the Cladocera. *Journal of Morphology* 256: 235–259.
- Pabst, T., and S. Richter. 2004. The larval development of an Australian limnadiid clam shrimp (Crustacea, Branchiopoda, Spinicaudata), and a comparison with other Limnadiidae. *Zoologischer Anzeiger* 243: 99–115.
- Panganiban, G., A. Sebring, L. Nagy, and S. Carroll. 1995. The development of crustacean limbs and the evolution of arthropods. *Science* 270: 1363–1366.
- Richter, S. 2002. The Tetraconata concept: hexapod-crustacean relationships and the phylogeny of Crustacea. *Organisms Diversity & Evolution* 2: 217–237.

- , and G. Scholtz. 2001. Phylogenetic analysis of the Malacostraca (Crustacea). *Journal of Zoological Systematics and Evolutionary Research* 39: 113-136.
- Romeis, B. (1989). *Mikroskopische Techniken*. 17te Auflage. Urban & Schwarzenberg, Wien. 697 pp.
- Scholtz, G. 1992. Cell lineage studies in the crayfish *Cherax destructor* (Crustacea, Decapoda): germ band formation, segmentation, and early neurogenesis. *Roux's Archives of Developmental Biology* 202: 36-48.
- . 1995. Expression of the *engrailed* gene reveals nine putative segment-anlagen in the embryonic pleon of the freshwater crayfish *Cherax destructor* (Crustacea, Malacostraca, Decapoda). *Biological Bulletin* 188: 157-165.
- . 2005. Homology and ontogeny: pattern and process in comparative developmental biology. *Theory in Biosciences* 124: 121-143.
- Schram, F. R. 1986. *Crustacea*. Oxford Press, New York. 606 pp.
- , and S. Koenemann. 2001. Developmental genetics and arthropod evolution: part I, on legs. *Evolution & Development* 3: 343-354.
- , and ———. 2004. Developmental genetics and arthropod evolution: on body regions of Crustacea. pp. 75-92. In, G. Scholtz (ed.), *Evolutionary developmental biology of Crustacea*. Crustacean Issues 15. A.A. Balkema, Lisse. 285 pp.
- Siewing, R. 1956. Untersuchungen zur Morphologie der Malacostraca (Crustacea). *Zoologische Jahrbücher, Anatomie und Ontogenie der Tiere* 75: 39-176.
- Spears, T., and L. G. Abele. 1999. Phylogenetic relationships of crustaceans with foliaceous limbs: an 18s rDNA study of Branchiopoda, Cephalocarida, and Phyllocarida. *Journal of Crustacean Biology* 19: 825-843.
- Thiele, J. 1905. Betrachtungen über die Phylogenie der Crustaceenbeine. *Zeitschrift für Wissenschaftliche Zoologie* 82: 445-471.
- Timms, B. V., and S. Richter. 2002. A preliminary analysis of the conchostracans (Crustacea: Spinicaudata and Laevicaudata) of the middle Paroo catchment of the Australian arid-zone. *Hydrobiologia* 486: 239-247.
- Ungerer, P., and C. Wolff. 2005. External morphology of limb development in the amphipod *Orchestia cavimana* (Crustacea, Malacostraca, Peracarida). *Zoomorphology* 124: 89-99.
- Waloszek, D. 1993. The Upper Cambrian *Rehbachella* and the phylogeny of Branchiopoda and Crustacea. *Fossils and Strata* 32: 1-202.
- , and K. J. Müller. 1998. Early arthropod phylogeny in the light of the Cambrian 'Orsten' fossils. pp. 185-231. In, G. Edgecombe (ed.), *Arthropod Fossils and Phylogeny*. Columbia University Press. 347 pp.
- Waloszek, D. 2003. The "Orsten" window - a three-dimensionally preserved Upper Cambrian meiofauna and its contribution to our understanding of the evolution of Arthropoda. *Paleontological Research* 7: 71-88.
- Williams, T. A. 1998. *Distal-less* expression in crustaceans and the patterning of branched limbs. *Development Genes and Evolution* 207: 427-434.
- . 2004. The evolution and development of crustacean limbs: an analysis of limb homologies. pp 170-193. In, G. Scholtz (ed.), *Evolutionary developmental biology of Crustacea*. Crustacean Issues 15. A.A. Balkema, Lisse. 285 pp.
- . 2007. Limb morphogenesis in the branchiopod crustacean, *Thamnocephalus platyurus*, and the evolution of proximal limb lobes within Anostraca. *Journal of Zoological Systematics and Evolutionary Research* 45: 191-201.
- , and G. B. Müller. 1996. Limb development in a primitive crustacean, *Triops longicaudatus*: subdivision of the early limb bud gives rise to multibranching limbs. *Development Genes and Evolution* 206: 161-168.
- Wolff, C., and G. Scholtz. 2008. The clonal composition of biramous and uniramous arthropod limbs. *Proceedings of the Royal Society B* 275: 1023-1028.
- Zhang, X., D. J. Siveter, D. Waloszek, and A. Maas. 2007. An epipodite-bearing crown-group crustacean from the Lower Cambrian. *Nature* 449: 595-598.

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