

The post-embryonic development of Remipedia (Crustacea)—additional results and new insights

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Abstract The post-embryonic development of a species of the enigmatic crustacean group Remipedia is described in detail for the first time under various aspects. Applying a molecular approach, we can clearly prove the species identity of the larvae as belonging to *Pleomothra apletocheles*. We document the cellular level of several larval stages and the differentiation of segments, limbs, and the general body morphology applying the techniques of confocal laser scanning microscopy and scanning electron microscopy. In addition, we document the swimming behavior and the peculiar movements of the naupliar appendages. A comparison of our results with published data on other Crustacea

and their larval development tentatively supports ideas about phylogenetic affinities of the Remipedia to the Malacostraca.

Keywords Arthropod evolution · Nauplius larva · Anamorphic development · Swimming behavior · Limb formation

Introduction

There are numerous studies of embryonic and larval development in crustaceans that have contributed to our understanding of the phylogeny and evolution of Crustacea and Arthropoda in general (Anderson 1973; Walossek 1993; Scholtz 2004). For example, the characteristic shape of the nauplius of the Branchiopoda shows several valid apomorphies for this group (Olesen 2007). The same is true for features of cirripede nauplii such as the frontolateral horns (Høeg et al. 2004). Malacostraca show distinct apomorphic cell division patterns involved in germ band and segment formation that are not found in other crustacean groups (Dohle et al. 2004). Within malacostracans, ontogenetic characters support the recognition of monophyletic groups such as amphipods (Scholtz and Wolff 2002) or freshwater crayfish (Scholtz 2002).

Remipedia is an enigmatic group of subterranean crustaceans that was discovered in an anchialine cave system on Grand Bahamas Island in 1979 (Yager 1981). Since then, several aspects of the group have been studied, and a number of new species east and west of the Atlantic Ocean have been described. Nevertheless, none of the phylogenetic analyses using morphological or molecular data resulted in a conclusive hypothesis about the phylogenetic position of remipedes within or even outside Crustacea (e.g., Schram 1986; Moura and Christoffersen 1996; Schram and Hof

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1998; Spears and Abele 1997; Wills 1998; Ax 1999; Richter 2002; Schram and Koenemann 2004; Fanenbruck et al. 2004; Giribet et al. 2005; Regier et al. 2005). Moreover, reconstructions of the internal relationships of Remipedia are currently only based on external adult features that may represent reductions or convergent adaptations to the life in anchialine caves (Koenemann et al. 2007b). Other morphological characters are not well-studied or unknown for the majority of taxa, including, for example, ultrastructure or ontogenetic data, the latter of which were completely unavailable for Remipedia until recently.

Accordingly, the discovery of some larvae in an anchialine cave systems on Abaco Island, Bahamas, in March 2006 represented a landmark in remipede biology (Koenemann et al. 2007a). However, due to the lack of material, this first description dealt only with some external features and had to remain superficial in many respects. The collection included one orthonauplius, three metanauplii, and a pre-juvenile (termed “post-nauplius” by Koenemann et al. 2007a). Based on the unique combination of external morphologic features, the larvae could be identified as Remipedia. However, the species identity of these larvae has remained doubtful since observations of complete life cycles were lacking.

A second cave diving expedition to the same caves on Abaco Island in August 2006 yielded nine additional nauplii of Remipedia. This new collection enabled us to study the morphology of larval stages in more detail using histology, fluorescence dyes in combination with confocal laser scanning microscopy and computer-aided 3D reconstruction and scanning electron microscopy (SEM). In addition, a comparative molecular sequence analysis of the mitochondrial marker cytochrome c oxidase subunit I (COI) was carried out to determine to which species of Remipedia the larvae belong.

We can show that the nauplii represent larval stages of *Pleomothra aplescheles*, the adults of which inhabit the same caves. The putative relationships to other crustacean groups are discussed in the light of our findings about the larval morphology of Remipedia at the cellular and morphogenetic levels. Although some characters support a close affinity to malacostracan crustaceans, the position of Remipedia within the Crustacea still remains to be solved.

Materials and methods

The nine additional larvae investigated in this study were collected in August 2006 and comprise two orthonauplii (body lengths, 155 and 157 μm) and seven metanauplii (167, 178, 184, 188, 192, 204, and 218 μm). The external morphology generally accords with that of the previous collection of nauplii from March 2006 (Koenemann et al. 2007a).

Video recordings of living larvae

Larvae were collected individually by hand in clear glass vials along with ambient water and kept cool during transfer back to the field lab, where microscopic examination and photography were carried out, typically within 4–6 h after collection. Still and video photography were done using a Cannon PowerShot G7 10 megapixel camera mounted on a trinocular Leica S6D stereozoom microscope with a 150-W fiber optic illuminator. Since the larvae are highly active swimmers, they were chilled in a Petri dish with cave water in a refrigerator for approximately 10–15 min before photography so as to slow them down and allow them to remain in frame. The mechanics of their swimming movements slowed markedly but were otherwise relatively unchanged by the cooling, and specimens typically recovered full mobility after gradual warming. As has been observed with live adult remipedes (Koenemann et al. 2007c), the larvae characteristically swim with their ventral side up using vigorous, coordinated movements of their mandibles (Fig. 6, see also video as [Supplementary material](#)).

SEM photography

For SEM photography, we used four nauplii that represent different developmental stages. Two specimens, collected during the first cave diving expedition in March 2006 (Koenemann et al. 2007a), included a 172 μm metanauplius (MN-1) and the larger pre-juvenile stage (“PN”; 370 μm). Two additional specimens prepared for SEM photography were collected during the second expedition in August 2006, an orthonauplius (ON-2, 157 μm) and a metanauplius (MN-4, 188 μm).

Since all four larvae were preserved in 100% ethanol, it was necessary to hydrate the specimens in a graded ethanol series and subsequently fix them in 1% osmium tetroxide aqueous solution for 30–45 min. After fixation, the specimens were dehydrated in a graded ethanol series and then critical-point dried in acetone using a Bal-Tec 030 CPD. The dried specimens were mounted on SEM stubs, sputter-coated with platinum–palladium following standard procedures and examined in a JEOL JSM-6335-F SEM. All SEM images were processed as digital images.

Fluorescence staining and confocal laser scanning microscopy

Two larvae (ON, 166 μm , described by Koenemann et al. 2007a; MN-5 167 μm) preserved in 70% ethanol were hydrated by adding 50% ethanol. For visualization of cell nuclei, specimens were washed 3×10 min in $1 \times$ phosphate-buffered saline (PBS; 1.86 mM NaH_2PO_4 , 8.41 mM

Na₂HPO₄, 175 mM NaCl in dH₂O; pH 7.4) and incubated in the DNA-specific Hoechst (H33258, Sigma) solution (1 µg/ml in PBS) for 10 min. Subsequently, unbound dye was washed out by several rinses with PBS (3×10 min), and the larvae were transferred to and mounted either in 2.5% DABCO-glycerol or Vectashield (Vector Laboratories).

As an alternative to Hoechst, the nucleic acid stain Sytox[®]Green (Molecular Probes) was used. Specimens were washed in phosphate-free Tris-buffered saline (TBS; 0.9% NaCl, 10 mM Tris-HCl; pH 7.5) several times (2×5 min, 4×30 min) before incubation in Sytox solution (5 nmol/ml in TBS) for 3 h. After subsequent washing in TBS (2–6×10 min, 4×30 min), larvae were transferred to and mounted in 2.5% DABCO-glycerol.

Digital images were taken under a fluorescence microscope (Zeiss Axiophot 1) and a laser scanning microscope (Leica SP2) using a Nikon D1. The laser-scanned image stacks were analyzed with the software Imaris 5.5.3 (Bitplane AG).

DNA sequence analysis

For DNA analysis, we used a metanauplius (MN-2; described by Koenemann et al. 2007a) that was collected from an anchialine cave system on Abaco Island, where four species of Remipedia are known to occur in sympatry: *Cryptocorynetes haptodiscus*, *Godzillionomus frondosus*, *Pleomothra apretocheles*, and *Speleonectes benjamini*. DNA was extracted from the metanauplius and adult specimens of each of the four species using a Qiagen QIAamp DNA Micro Kit. All samples were shock-frozen in liquid nitrogen and crushed with a pestle. Extracts were resuspended in 30 µl Tris-EDTA buffer. Extracted DNA was unspecifically amplified using a Qiagen Repli-g Mini Kit. A fragment composed of 569 bp was amplified at the 5' end of COI with the primers *mtCOI-149s0* *ggcaacaatacataaaga tattgg* and *mtCOI-2198* *taaacttcagggtgaccaaaaaatca* (Folmer et al. 1994). A polymerase chain reaction (PCR) was conducted with an Eppendorf ep Gradient Cycler using 25 µl reaction volume containing template, 2.5 µl 10× PCR buffer (Bioline), 2 mM MgCl₂ (Bioline), 10 pmol of each primer (Biomers), 0.4 mM deoxyribonucleotide triphosphate (dNTP; Bioline), 1 U Taq (Bioline), and 2% DMSO (Roth), with an initial denaturation 3 min at 94°C; 40 cycles for 30 s at 94°C, 50 s at 50°C, and 1 min at 72°C and final elongation for 5 min at 72°C. PCR products were visualized on a 1.4% agarose gel containing 1% ethidium bromide; samples showing clear bands were purified using a Qiagen Qiaquick PCR Purification Kit and bidirectionally sequenced by Macrogen (Korea). Sequences were assembled in Seqman II (Lasergene), and pairwise distances of the alignment were calculated with the Mega 3.1 software program (Kumar et al. 2004).

Results

Ecological aspects

Larvae were collected by divers using Megalodon-closed circuit rebreathers at distances of 500 m or more inside two completely submerged anchialine caves on Abaco, Bahamas. These caves are situated in pine forest in the interior of the island, about 3 km from the nearest coastline. They are part of an extensive cave system consisting of large, breakdown chambers interconnected by clay floored passages at 45–50 m depths. Massive speleothems (i.e., stalactites and stalagmites) are abundant in the larger chambers, bearing witness to prolonged periods of lower glacial sea level when the caves were dry and air-filled. The cave water column is highly stratified with freshwater extending down to 12 m depth, followed by a well-defined halocline terminating in fully marine water at 17 m depth (Fig. 1). Water temperature dropped abruptly in surface water and then more gradually with depth, reaching a minimum of 23.5°C in marine waters. Both dissolved oxygen and pH showed significant decreases at the halocline, reaching minimum values of 2.68 mg/l and 6.64, respectively. The larvae were observed swimming in the water column, just below the halocline in fully marine water at 18–20 m depths. While adult remipedes were randomly and widely distributed in marine cave waters, the larvae were clustered primarily at these depths and appeared to be localized in specific regions within the cave. Since the cave water is exceptionally transparent with no visible particulate matter, even small animals such as the larvae show up well in the beam of an HID dive light. Before collection in individual vials, the swimming behavior of the larvae was observed to be essentially the same as that documented in lab Petri dishes.

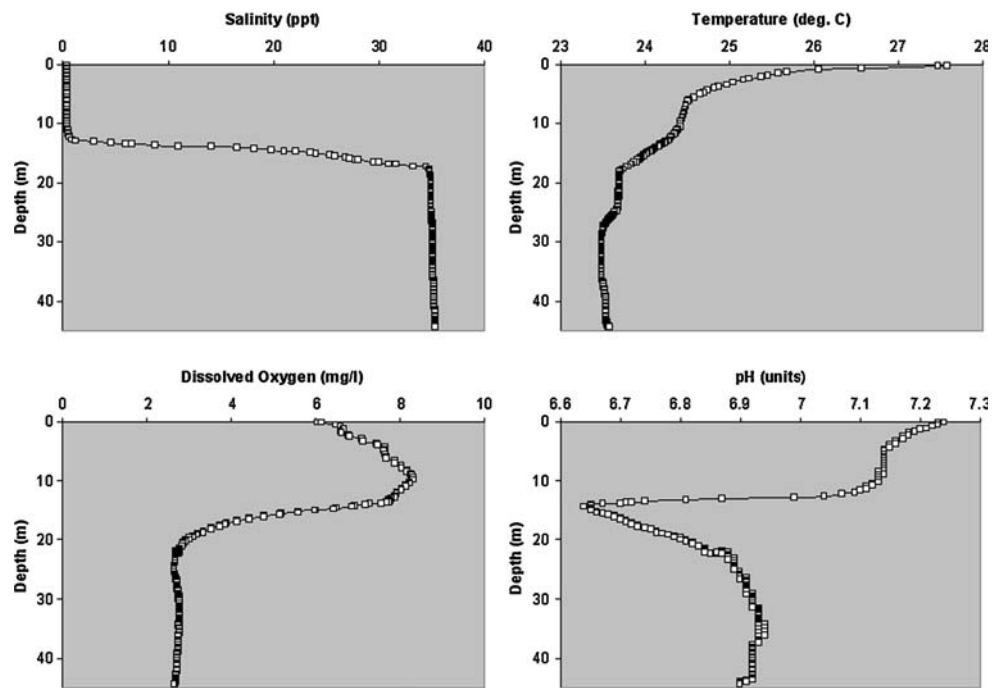
DNA sequence analysis

A comparison of a 569-bp fragment of COI shows no variation between the sequences of a larva (MN-2) and an adult specimen of *P. apretocheles*. Sequence variation between the larva and the other remipede genera ranged from 0.20 to 0.40 (Table 1). Based on these COI sequence data, we can confidently classify the larvae as belonging to the species *P. apretocheles*. This result supports the previous classification of remipede nauplii based on morphological characters (Koenemann et al. 2007a; Koenemann et al. 2008).

Orthonauplius

The fluorescent staining of the smaller one of the two nauplii (ON) reveals a number of post-naupliar segment anlagen that are covered by a cuticle (Fig. 2a). Therefore,

Fig. 1 Vertical profiles of salinity, temperature, dissolved oxygen, and pH as measured with a YSI 600 XLM water quality logger deployed by a diver. Measurements were taken at 2-s intervals during descent from the surface to the deepest accessible point in the cave. Individual data points are shown as open squares



this stage is strictly spoken not a proper orthonauplius (Figs. 2, 4) as has been suggested by Koenemann et al. (2007a). With a maximum body length of 166 μm and a width of 80 μm , this larva is only slightly smaller than “true” metanauplii with visible, external trunk limb buds (see below and Koenemann et al. 2007a).

The larva contains a copious amount of yolk and is covered mostly by large flattened cells. The distances between nuclei are comparatively large on the dorsal side and along the lateral body regions (Fig. 2b). On the ventral side, more densely packed cells forming several layers indicate the differentiating embryo, in particular in the posterior (post-naupliar) region (Fig. 2a). The uniramous first antenna is divided into a basic peduncular section followed by a slender distal part that exhibits an incipient articulation (see also Fig. 4c). The second antennae and the mandibles are biramous appendages. The basal part of the mandible shows a bipartite area of condensed cells forming the anlage of the gnathal part (Figs. 2a, c). In the spherical naupliar region, the larval brain anlagen can be identified, beginning with the (protocerebral) paired head lobes at the very anterior end of the larva (Figs. 2a, d). The deutocerebrum anlage forms a pair of distinct lobes posterior to the bases of the first antennae. The tritocerebrum anlage is not well defined; it is composed of small cell clusters posterior to the deutocerebrum and appears between the bases of second antennae and mandibles (Fig. 2c). The unpaired labrum anlage is situated between the paired tritocerebrum primordia anterior to the stomodaeum, which is recognizable as a median region of relatively few cells

separating the ganglia of second antennae and mandibles (Figs. 2a, c, d).

The post-naupliar region shows on its ventral side at least five visible segment anlagen—the segments of first and second maxillae, maxillipeds, and two trunk segments—indicated by paired primordia of ganglia and limb buds that exhibit a decreasing degree of differentiation from anterior to posterior. At this stage, all limb buds are undivided and point postero-ventrally (Fig. 2a), but already in ON-2, the buds of the first trunk limbs are subdivided into anlagen of the endopod and exopod (see Fig. 4i). Between the ganglion

Table 1 Pairwise distances of COI sequences between all species of Remipedia that are known to inhabit the cave system from which the larvae were collected

	1	2	3	4	5
1. Larva (FJ527838)					
2. <i>Pleomothra aplectocheles</i> (FJ527840)	0.00				
3. <i>Cryptocorynetes haptodiscus</i> (FJ527837)	0.34	0.34			
4. <i>Godzillignomus frondosus</i> (FJ527839)	0.40	0.40	0.27		
5. <i>Speleonectes benjamini</i> (FJ527841)	0.38	0.38	0.20	0.29	
6. <i>Marsupenaues japonicus</i> (NC_007010)	0.43	0.43	0.35	0.35	0.38

Model: Kimura-2-parameter; gaps/missing data: complete deletion; substitution to include: transitions + transversions. The malacostracan *Marsupenaues* has been included for comparison; GenBank accession numbers are given in parentheses

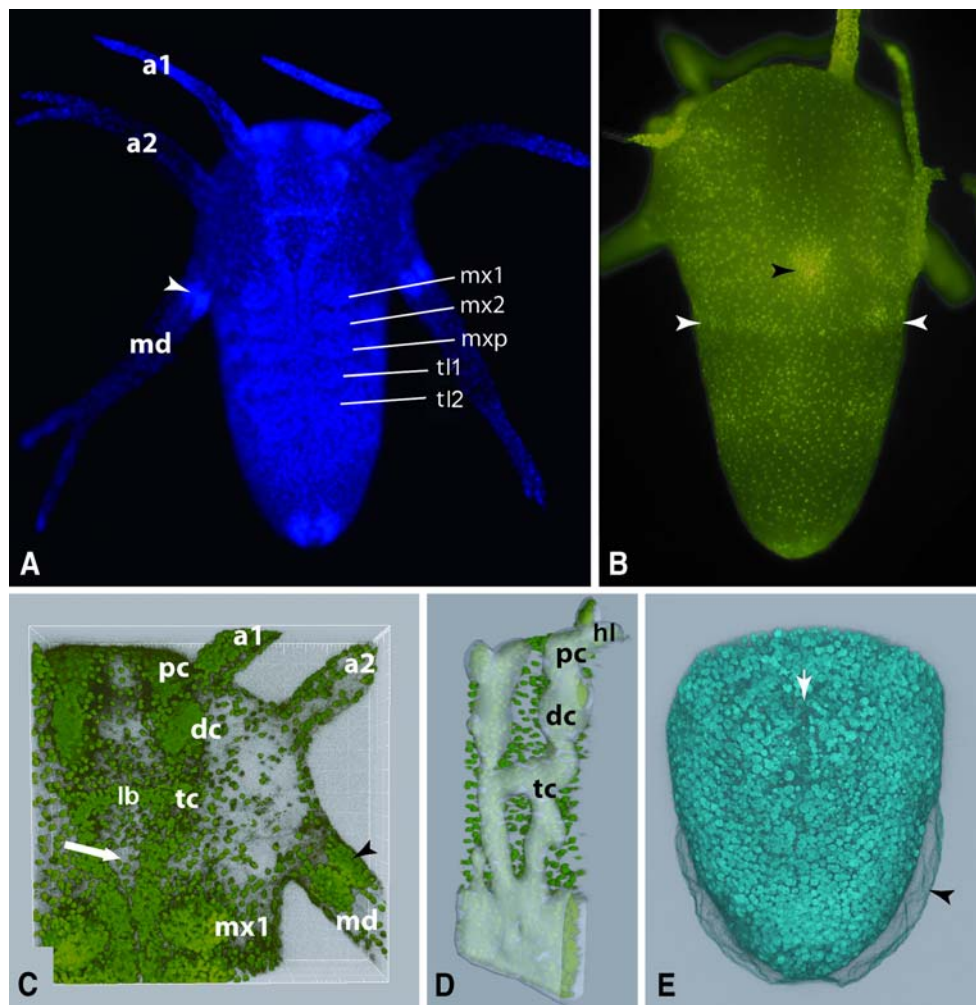


Fig. 2 Fluorescent staining of an orthonauplius (*ON*). **a** Ventral view revealing the limb bud anlagen of the first and second maxillae (*mx1*, *mx2*), the maxillipeds (*mxp*), and the first two pairs of trunk limbs (*tl1*, *tl2*); the second trunk limbs are just beginning to form. The *arrowhead* points to the bipartite area of densely arranged nuclei in the basal part of the mandible. **b** Dorsal view; the *black arrowhead* indicates a cluster of densely distributed cells that may represent the anlage of a dorsal organ; *white arrowheads* demarcate the transverse band of cells, indicating the posterior margin of the forming head shield. **c** Detail of the anterior spherical region, ventral view. The *black*

arrowhead points to the densely arranged nuclei in the basal part of the mandible. The *white arrow* marks the area of the forming stomodaeum. **d** 3D image visualization of the anterior spherical region with the brain anlagen (*transparent gray*). **e** Posterior part of the larva; the *white arrow* points to the midline that is formed by cells with nuclei lying slightly deeper than the other nuclei. Laterally, the cuticle is slightly detached (*black arrowhead*). *a1* first antenna, *a2* second antenna, *dc* deutocerebrum anlage, *lb* labrum anlage, *hl* head lobes, *md* mandible, *pc* protocerebrum anlage, *tc* tritocerebrum anlage

anlagen of the post-mandibular segments, there is a midline of unpaired cells lying somewhat deeper in the embryo (Figs. 2a, e). The posterior growth zone is characterized by densely packed, scattered cells without any regular arrangement and lacking a defined midline (Fig. 2e). The posterior-most region of the body is characterized by two outgrowths lateral to the forming proctodaeum, which represent the furca anlagen (Fig. 2a).

On the dorsal side of the larva, the distribution of cells forms two particular patterns. Slightly posterior to the mandibles, there is a median arrangement of densely packed nuclei, which probably form the anlage of a dorsal organ (Fig. 2b). The second conspicuous structure is a transverse

band of nuclei directly posterior to the putative dorsal organ, approximately between the first and second maxillary segments (Fig. 2b). This position corresponds with the posterior margin of the forming head shield (Fig. 2b).

Metanauplius

The advanced larvae are only slightly longer but slimmer than the orthonauplii; their body lengths range from 167 to 218 μm , with a maximum width of 71 μm (Figs. 3 and 4). Compared to the orthonauplius, the number of cells on the ventral side has strongly increased (Fig. 3a). The naupliar limbs appear more or less unchanged. However, the

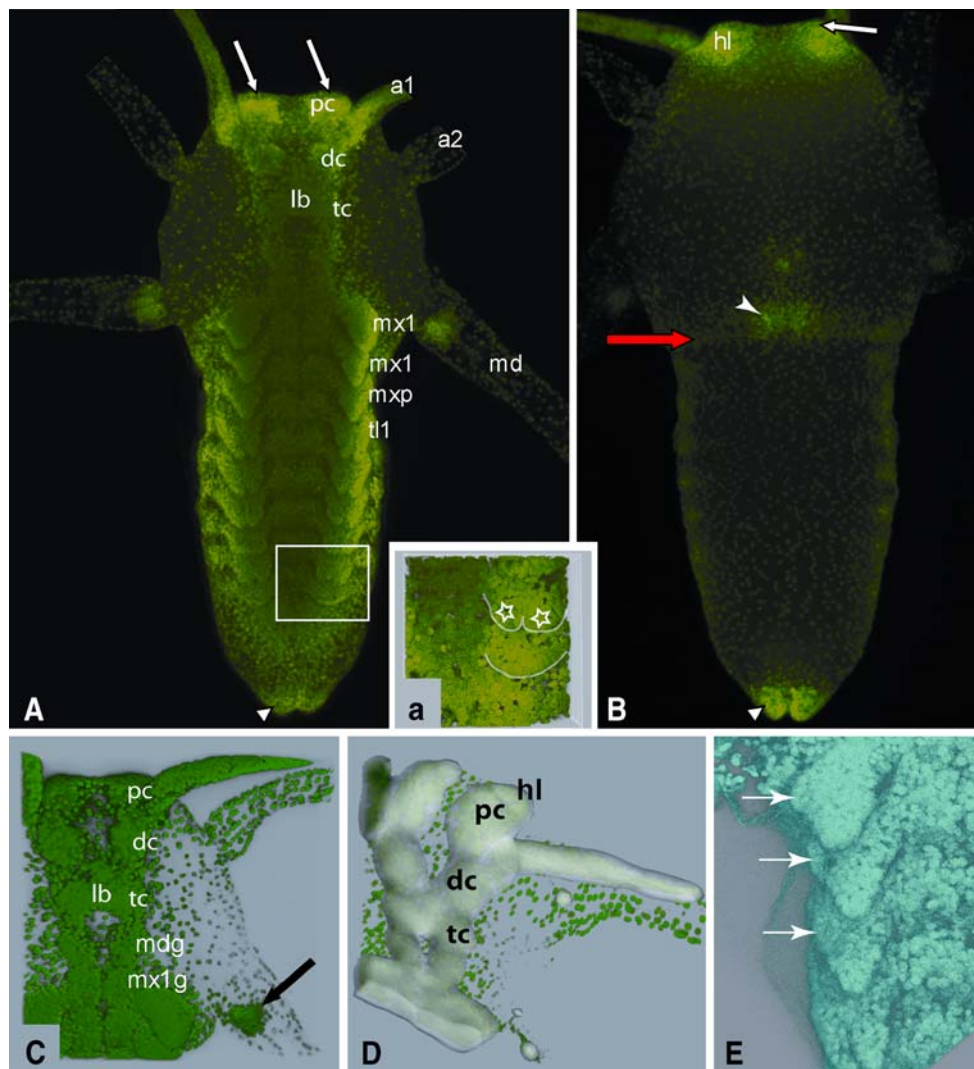


Fig. 3 Fluorescent staining of a metanauplius (*MN-5*). **a** Ventral view showing the nervous system and the appendage anlagen of the naupliar and the post-naupliar region. The *arrows* point to the forming frontal filaments at the inner margins of the protocerebral (*pc*) head lobes. The *white triangle* marks the forming furcal rami. The *insert* in **a** reveals the bi-lobed limb bud of the fifth trunk segment (indicated by *asterisks*) in contrast to the following less advanced undivided limb anlage of the sixth trunk segment. **b** Dorsal view; the *white arrow* indicates the position of the putative right frontal filament anlage, the *arrowhead* points at the presumed anlage of the dorsal organ; the *red arrow* marks the transverse band of cells forming the posterior margin of the head shield; the *white triangle* marks the forming furcal rami. **c** Ventral view of the head region revealing the brain anlagen. The

arrow points to the cell cluster at the basal part of the mandibles. **d** 3D image of the same region shown in **c** visualizing the main parts of the brain anlagen. **e** Detail of post-mandibular body region (ventral view); *arrows* point at vestigial bulges at the proximo-lateral margins of uniramous limb buds of first and second maxillae and maxilliped, probably representing exopod anlagen (from *top to bottom*), followed by a biramous limb bud of the first trunk limb. *a1* first antenna, *a2* second antenna, *dc* deutocerebrum anlage, *hl* head lobe, *lb* labrum anlage, *md* mandible, *mdg* mandibular ganglion, *mx1* first maxilla, *mx2* second maxilla, *mx1g* ganglion of the first maxilla, *mxp* maxilliped, *pc* protocerebrum anlage, *tc* tritocerebrum anlage, *tl1* first trunk limb

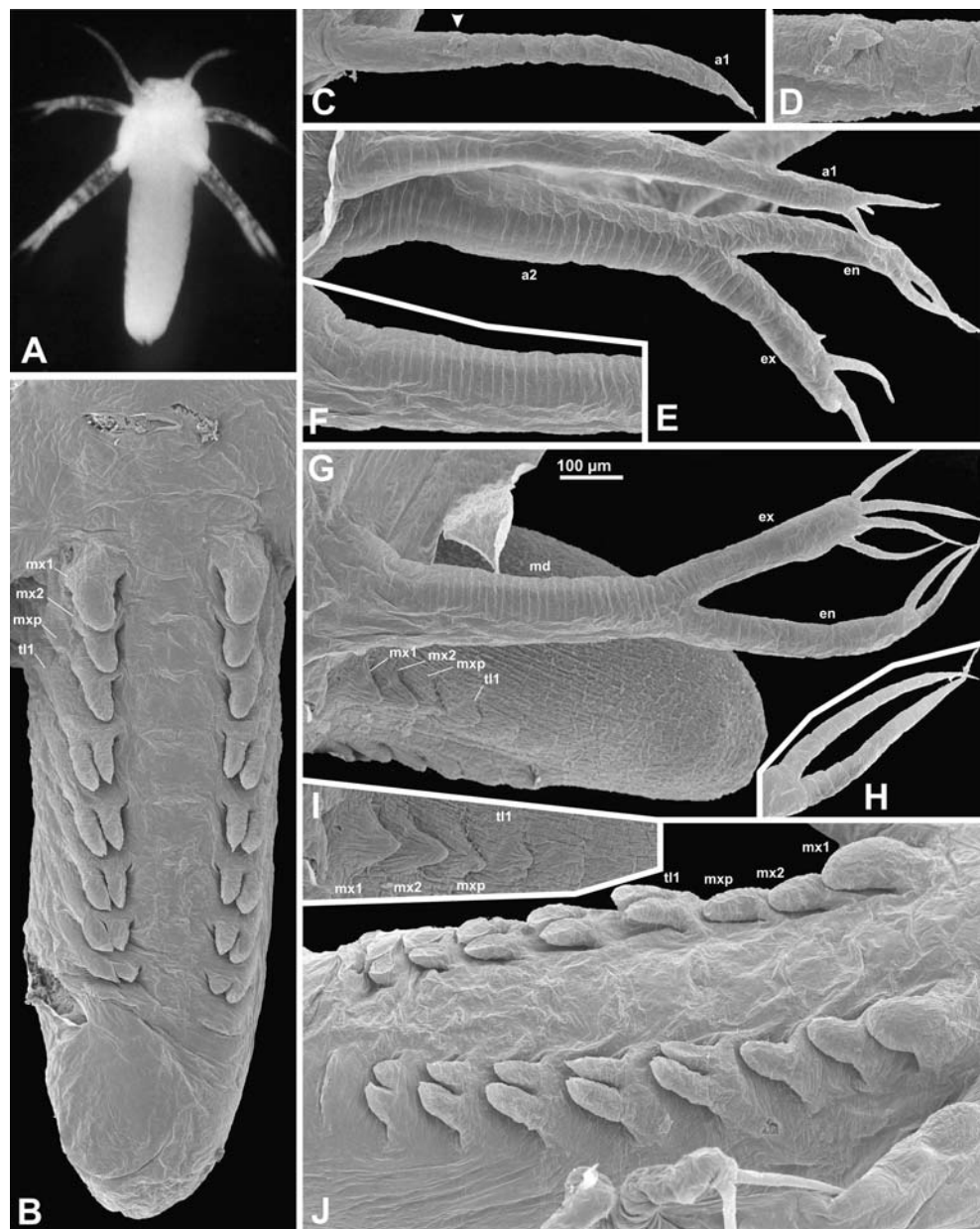
putative anlage of the mandibular gnathobase is relatively smaller, and the bipartition of the dense cell clusters has disappeared (Fig. 3c).

The brain appears generally further developed and is more clearly recognizable (Figs. 3a–d). The protocerebral part with its head lobes reaches now to the dorsal side (Fig. 3b); at the anterior margin of the prospective head region, between the first antennae, paired bulged structures have developed that presumably will give rise to the frontal

filaments (Figs. 3a, b). The deutocerebrum anlage forms two massive, round structures (Figs. 3c, d). The tritocerebrum anlage now includes par-oral cell clusters, and the mandibular ganglia have become distinguishable structures (Fig. 3c). Both labrum anlage and invagination of the stomodaeum are somewhat more distinct.

There is a small pointed scale on the mid-proximal part of the first antenna that represents the developing ventral flagellum (Figs. 4c, d).

Fig. 4 SEM images of an ortho-nauplius (*ON-2*) and metanauplius (*MN-4*). **a** Photo of a living larva *MN-4*. **b** Posterior body region of *MN-4* (ventral view). **c** First antenna of *MN-4* (arrow points at scale-like ventral ramus). **d** Detail of first antenna of *MN-4*, with enlarged scale-like ventral ramus. **e** Antennae 1 and 2 of *ON-2*. **f** Proximal region of mandible of *ON-2*. **g** Mandible of *ON-2*. **h** Distal setation of mandibular endopod of *ON-2*. **i** Early limb buds (*mx1*, *mx2*, *mxp*, *tl1*) of *ON-2*. **j** Posterior body region of *MN-4* (ventro-lateral view). *a1* first antenna, *a2* second antenna, *en* endopod, *ex* exopod, *md* mandible, *mx1* first maxilla, *mx2* second maxilla, *mxp* maxilliped, *tl1* trunk limb 1



The ventral nervous system has more massive and multilayered ganglia anlagen. These form paired segmental swellings, which are medially separated by the distinct population of the deeper lying midline cells (Fig. 3a). The fusion of the ganglia of the mandibles, the two pairs of maxillae, and the maxilliped known from adult *Remipedia* (Fanenbruck et al. 2004; Fanenbruck and Harzsch 2005) has not yet taken place at this stage. All nine visible post-naupliar limb buds point to the posterior. The buds of the first and second maxillae and of the maxillipeds are elongated and uniramous. However, small vestigial bulges at the outer proximo-lateral margins of these three limbs probably represent the anlagen of the exopods (Fig. 3e). The trunk limbs appear as biramous buds, with two similar branches forming the anlagen of the exopod and the endopod

(Figs. 3a, e, and 4b, j). Only the posterior-most small pair of trunk limb buds is still undivided (Figs. 3a, and 4j).

On the dorso-lateral side, each post-naupliar segment contains groups of densely packed cells, which form the anlagen of the tergites (Fig. 3a, b). The transverse cell band of the head shield anlage on the dorsal side now comprises more cells; it marks the posterior margin of the head region, separating head and trunk in the area of the second maxillary segment (Fig. 3b). The anlage of the dorsal organ has moved posteriorly and is partly fused with the transverse cell band.

The posterior end of the larva is characterized by the paired furca anlagen that have grown slightly longer and moved, together with the proctodaeum, to a more dorsal position (Figs. 3a, b).

The pre-juvenile stage

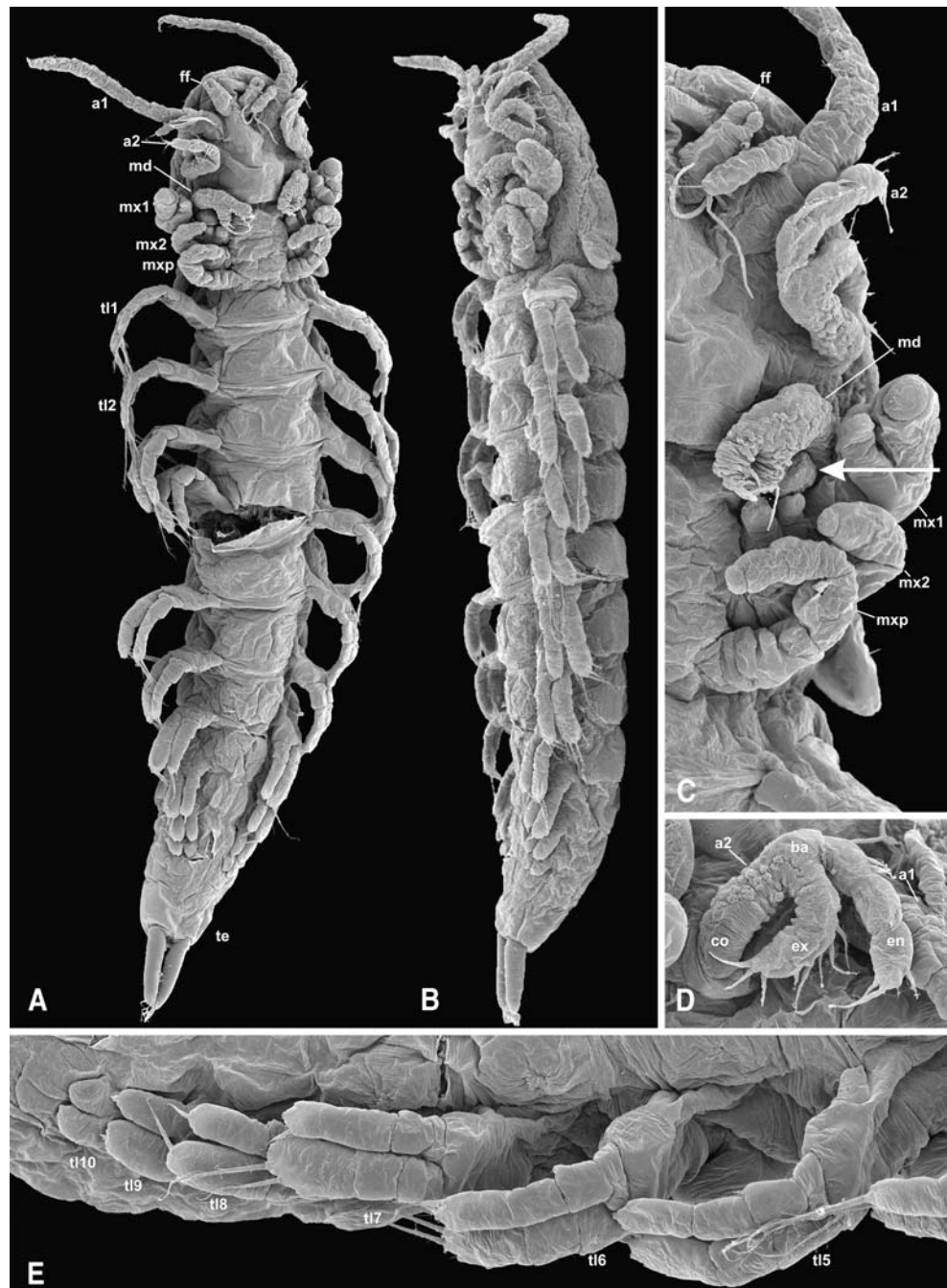
The only pre-juvenile specimen (first treated by Koenemann et al. 2007a, 2008) collected until now has been re-examined using SEM (Fig. 5). The specimen is about 3.75 mm long and has a general adult appearance but with fewer body segments, fewer trunk limbs, undeveloped cephalic appendages, and several retained larval features. Swimming has not been observed, but the limbs mainly responsible for locomotion are obviously the anterior trunk

limbs, and the locomotion therefore must resemble that of adults (see Kohlhage and Yager 1994; Koenemann et al. 2007c).

The paired, bifurcate frontal filaments anterior to the first antennae are, compared to the adult, relatively large. The main branch of the first antenna is divided into 12 segments, of which the proximal segment is the largest; a short, yet undivided ventral branch inserts on the second segment.

The second antennae are still biramous but probably no longer contribute to locomotion. The orientation and basic

Fig. 5 SEM images of a pre-juvenile. **a** Ventral view. **b** Lateral view. **c** Cephalic appendages, left side (ventral view). **d** Second antenna. **e** Trunk limbs 5 to 10, left side (ventral view). *a1* first antenna, *a2* second antenna, *ba* basis, *co* coxa, *en* endopod, *ex* exopod, *ff* frontal filaments, *md* mandible, *mx1* first maxilla, *mx2* second maxilla, *mxp* maxilliped, *tl1–10* trunk limbs 1–10. The *arrow* points to the anlage of the mandibular gnathobase



morphology of the second antennae has changed so that the stems of the limbs have bent ventrally and the rami turned dorsally. In general, the second antennae attain the adult condition, but the naupliar morphology is still recognizable. The stem of the second antennae is weakly subdivided into coxa and basis, the endopod into three segments, while the exopod is undivided.

The mandible is still incompletely developed and has retained the rudimentary rami of the exopod and endopod. These rami have bent ventrally; they appear nonfunctional at this stage and are not involved in locomotion. The mandibular gnathal processes are only weakly developed at this stage and not functional (Fig. 5c).

The single specimen examined displays serial trunk limbs developed to different degrees, which, in this way, illustrate the development of a single limb (Fig. 5e). Ten trunk limbs (tl) are “free” from the body, and the anlagen of two posterior-most trunk limbs can be identified as marks in the cuticle. The limbs first appear as paired ventral anlagen, corresponding to the future endopod and exopod. The endopod and exopod become “free” from the body as pairs of undivided limb buds before the stem of the limb is clearly visible (tl 9–10). The four segments of the endopod and the three segments of the exopod are formed gradually in a strict proximodistal sequence in each ramus (tl 5–8). The relative sequence of the segmental subdivision of the endopod in relation to the exopod is as follows: 1/1, 2/1, 3/2, 3/3, and 4/3 (see Fig. 5). Only the smallest limb buds are in an approximate ventral position (tl 8–10), while the more anterior limbs appear in a more or less lateral position as in the adults.

Movability of naupliar appendages

We could not detect any external division into coxal and basipodal regions in the 2nd antennae or the mandibles (Figs. 2, 4e–g). SEM photographs reveal numerous superficial sutures accompanied by rows of minute cuticular denticles along the entire length of the naupliar limbs (first and second antennae and mandibles; Fig. 4e–g). The exopod bears four bare setae, three terminal and one slightly ventral (Fig. 4g). The endopod is equipped with two terminal bare setae (Fig. 4h). Despite the apparent lack of limb articulation, video recordings of living ortho- and metanauplii in Petri dishes show that at least the second antennae and the mandibles are rather flexible (Koenemann et al. 2007c; Fig. 6).

During swimming (Fig. 6a), the first antennae are mostly directed forward and only perform feeble asynchronous strokes that are most likely insignificant for locomotion. In contrast, the second antennae and mandibles are very active, and both limbs apparently contribute to locomotion despite bearing only few, bare distal setae (suggesting a weak grip in the water). The second antennae perform more

or less constant strokes that cover a range of about 180° lateral to the body axis. Most strokes of the second antennae are more or less synchronized, but independent movements are also seen, often resulting in a shift in swimming direction. The mandibles cover a range of movements from a position about 45° laterally to the main body axis to a position stretched out along the body in posterior direction. During the power stroke the mandibles are almost stretched out, while they are slightly bent during the recovery stroke. Moreover, the mandibles exhibit asynchronous movement patterns seemingly related to a shift in direction (Fig. 6a).

The swimming described above was based on video sequences of individuals that were moving freely around in the Petri dishes in a manner close to what could be observed directly in the cave. Other types of limb movements were also documented, but it is uncertain how frequent these are performed under natural conditions. For example, the mandibles often bend over the ventral trunk so that the two rami meet in a “folded hands” position, and even the two rami can move independently as they occasionally move toward each other like a pair of forceps (Fig. 6b). Apart from the insertion point of the mandibles on the trunk, there are no specific joints or distinct articulations; the limbs rather appear to be flexible along the whole length. The second antennae are similarly flexible and also have rami that can move independently from each other. The flexibility of the second antennae certainly has at least partly a cleaning function, since some video sequences show them cleaning the first antennae (Fig. 6c). The cleaning typically takes places the following way: While the first antenna is bent laterally or posteriorly along the body, it is caught by the flexible second antenna, which then cleans the first antenna from proximal to distal in a sweeping motion. Sometimes, the first antenna is even dragged between the two rami of the second antenna (Fig. 6c). The second antennae have also been seen cleaning parts of the mandibles (see [Supplementary material](#)).

Discussion

Larval cycle of Remipedia

Due to the still limited number of remipede nauplii we have at hand, the larval sequence is most likely not fully understood. However, our examination of a total of 14 larval specimens indicates that the development proceeds in a comparatively gradual pattern. Based on our current knowledge, we can distinguish three larval categories: (1) orthonauplius (postnaupliar limbs absent externally, see above), (2) metanauplius (with postnaupliar limb buds, but naupliar limbs still present), (3) pre-juvenile phase (naupliar

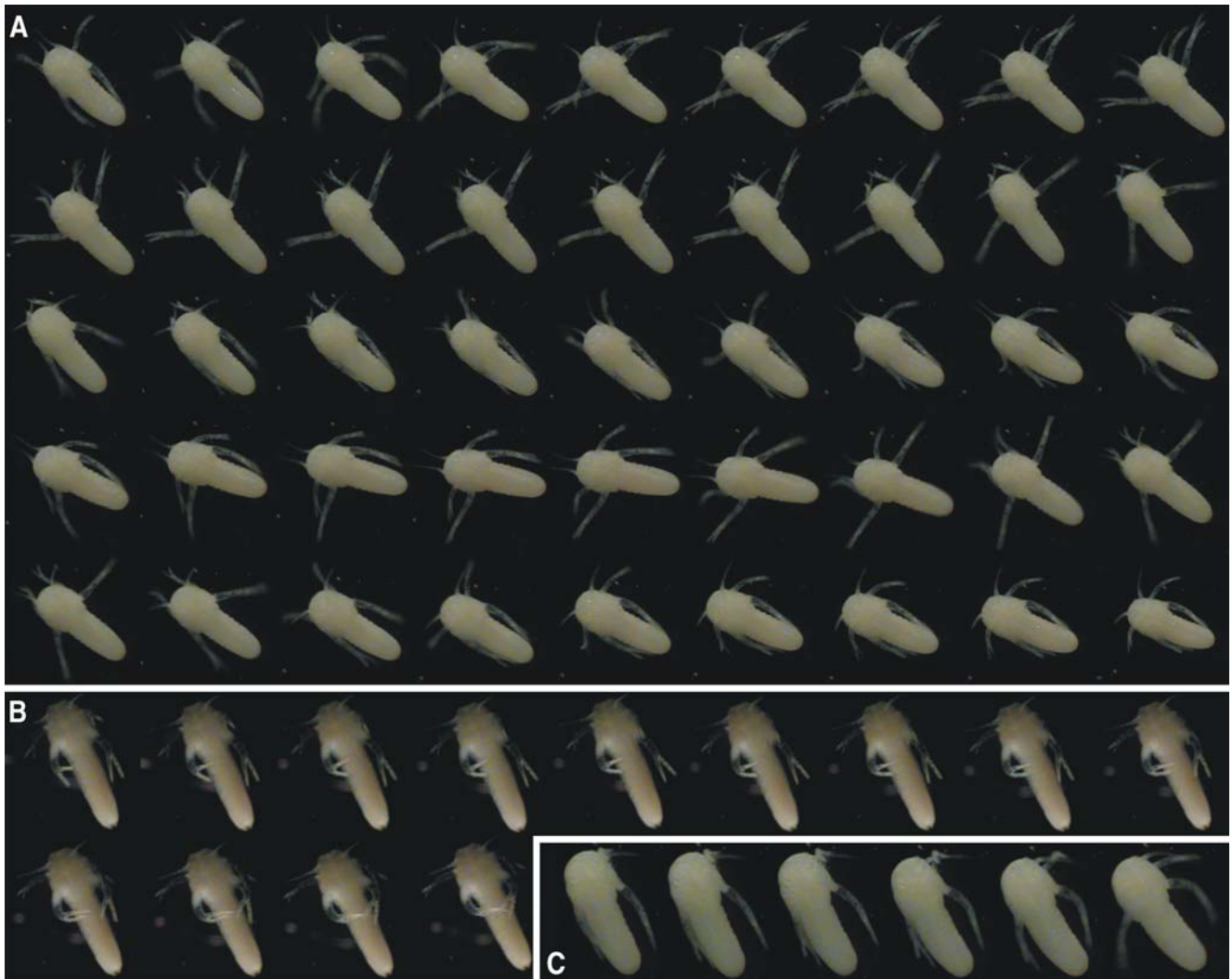


Fig. 6 Video snapshots of a moving remipede metanauplius illustrating swimming behavior, flexibility, and mobility of the naupliar appendages. Animals were filmed in a Petri dish with cave water. **a** Swimming sequence with two complete limb cycles shown. **b**

Flexibility and mobility of the mandibles. **c** Sequence showing the second antenna cleaning the first antenna (see video provided as [Supplementary material](#))

limbs present as rudiments only, locomotion transferred to trunk limbs). During these three phases, remipede larvae are lecithotrophic. In the subsequent developmental phase, the naupliar limb rudiments are transformed, and the immature, juvenile remipedes begin to feed.

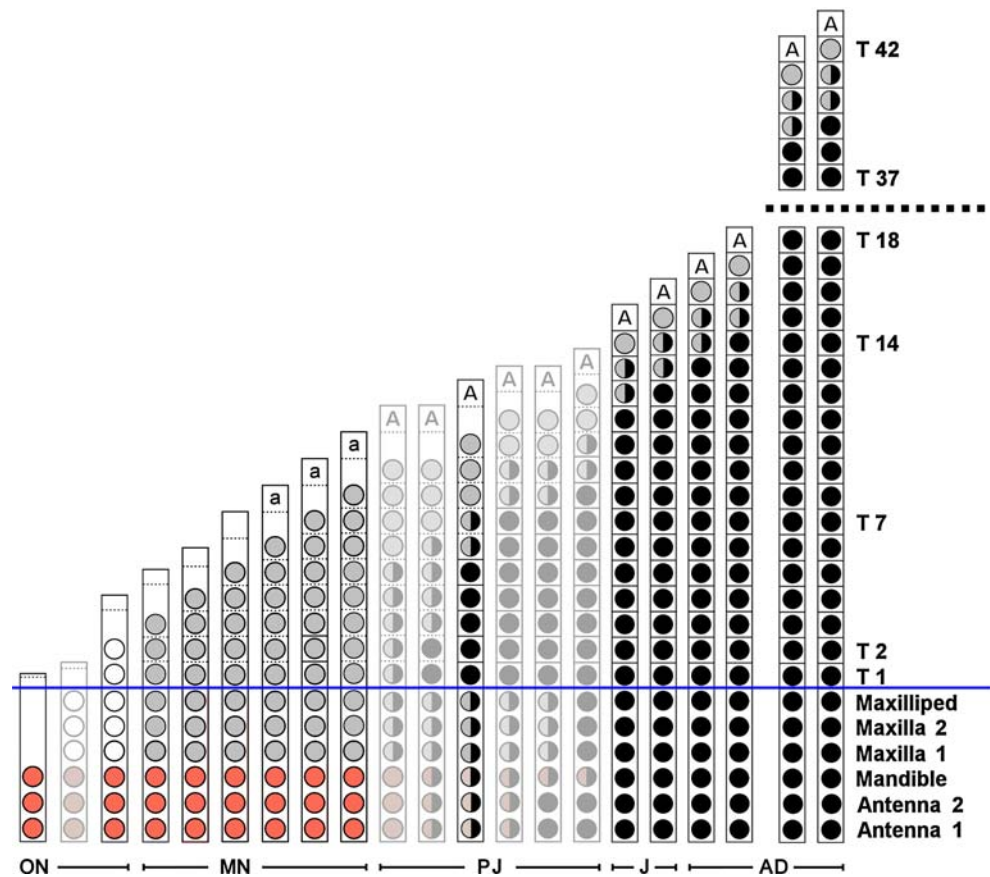
Based on the known larvae, the development is anamorphic, with only few changes between subsequent stages (summarized in Fig. 7, where a few hypothetical stages are indicated as shaded columns). In the earliest stage, only the naupliar appendages are visible externally. The next developmental stage still bears the naupliar appendages, but the larva is longer, and anlagen of about five limbs are developing under the cuticle (Fig. 4g, i).

Six metanaupliar stages have been discovered, which largely differ only with respect to body length and the number of developing trunk limbs (see Figs. 3, 4, and 6). As shown in

Fig. 7, both body segmentation and the number of trunk limbs increase stepwise, and the development therefore seems to proceed in an anamorphic pattern. There is a “developmental gap” between the most advanced metanaupliar stage and the next stage discovered, the pre-juvenile. This stage is characterized by a basically adult mode of locomotion using the trunk limbs, while the naupliar appendages seemingly lost a locomotory function. We find another developmental gap between this pre-juvenile stage and later, true juveniles. Based on the presumed anamorphic development as seen in the metanauplii, we assume that some early (pre-)juvenile stages still need to be discovered (indicated as shaded columns in Fig. 7).

An anamorphic larval development is seen in a number of crustaceans such as Cephalocarida (Sanders 1963; Addis et al. 2007), Anostraca (Benesch 1969), Euphausiacea, and

Fig. 7 Developmental diagram of Remipedia. Early development based on *Pleomothra aplocheles* (ON to PJ), subsequent juvenile and adult stages represent Remipedia in general. *ON* orthonauplius, *MN* meta-nauplius, *PJ* pre-juvenile, *J* juvenile, *AD* adult, *A* anal somite, *a* incipient anal somite, *T* trunk limb (42 trunk segments is the maximum number recorded for Remipedia); *open circle* developing limb under cuticular membrane, *gray circle* incipient limb (bud), *filled circle* fully developed limb, *red circle* naupliar limbs, *half-filled circle* developing or transitional limb (articulation mostly lacking). Columns shaded with gray represent hypothetical developmental stages. The blue vertical line delineates the head-trunk boundary; bold dotted line indicates trunk segments between T18 and T37 that have been excluded for convenient layout



Dendrobranchiata among Malacostraca (Scholtz 2000) and also for the branchiopod “Orsten” fossil *Rehbachella kinnekullensis* (Waloszek 1993). However, compared to these taxa, our knowledge of the development of Remipedia is still incomplete. Therefore, it is too early to say whether remipede development takes a similarly anomorphic course or not. With respect to the extreme delay in the onset of food uptake, remipedes also differ from most of these crustaceans. In recent branchiopods, feeding starts after one to three stages, in Cephalocarida and *Rehbachella* apparently already during the first developmental stage. Even compared to crustacean taxa characterized by a lecithotrophic early development (e.g., rhizocephalan, penaeid, and euphausiid larvae), lecithotrophy in Remipedia appears to take an unusually long period of time, as even the examined pre-juvenile represents a non-feeding stage (Fig. 5).

Do larval structures help to resolve remipede affinities?

The phylogenetic position of Remipedia is far from clear. Often regarded as an early offshoot of crustaceans (e.g., Schram 1986; Brusca and Brusca 1990; Wills 1998; Ax 1999), grouped among “maxillopodan” crustaceans (Boxshall 1997), or even non-crustacean arthropods (Moura and

Christoffersen 1996), more recent studies on brain anatomy suggest a close affinity or sister-group relationship to Malacostraca (Fanenbruck et al. 2004). In molecular analyses, they appear sometimes as a sister-group to Cephalocarida, Copepoda, Cirripedia, Malacostraca, and even collembolan or dipluran hexapods (Regier et al. 2005; Giribet et al. 2005; Cook et al. 2005; Carapelli et al. 2007).

Remipedia as Crustacea

The monophyly of Crustacea is a contentious issue (e.g., Richter 2002; Waloszek 2003; Giribet et al. 2005). Several morphological analyses support crustacean monophyly (e.g., Schram and Hof 1998; Wills 1998; Edgecombe 2004; Giribet et al. 2005), but some morphological and virtually all molecular data sets result in paraphyletic Crustacea with closer affinities of one crustacean subgroup to hexapods or even a mutually paraphyly of Crustacea and Hexapoda (e.g., Schram and Koenemann 2004; Regier et al. 2005; Giribet et al. 2005; Cook et al. 2005; Carapelli et al. 2007). Hence, it is difficult to assign Remipedia to Crustacea. This is even more hampered by the fact that most putative crustacean apomorphies such as the nauplius eye (Lauterbach 1983) or characters of the larval mouth region (Waloszek 2003) do not occur in Remipedia. The nauplius larva with three

functional pairs of appendages, which is present in Remipedia, is considered an apomorphy of crown-group Crustacea (see Waloszek 2003). However, it might as well be a character of the Mandibulata in general (see Lauterbach 1983; Ax 1999; Richter 2002) rather than an indication for a clade Crustacea. Accordingly, the only method to circumvent this problem is to look for particular affinities and correspondences between Remipedia and other crustacean taxa.

Similarities to Malacostraca

In their ground pattern, malacostracan embryos and larvae are characterized by a number of developmental features not known from other crustacean taxa. These apparently apomorphic characters include (1) a ventrally folded caudal papilla, (2) a relatively low number of cells in the germ band in combination with a stereotypic arrangement and division pattern of these germ band cells, (3) a growth zone comprising a distinct number of ectodermal and mesodermal teloblasts, and (4) a distinct population of midline cells forming the mirror axis of the germ band (Scholtz 2000; Dohle et al. 2004). Moreover, a limb formation with laterally oriented initial limb buds has been found in leptostracans (Williams 1998; Olesen and Walossek 2000; Pabst and Scholtz 2009), and it seems sensible to assume this feature as a plesiomorphic condition within the Malacostraca.

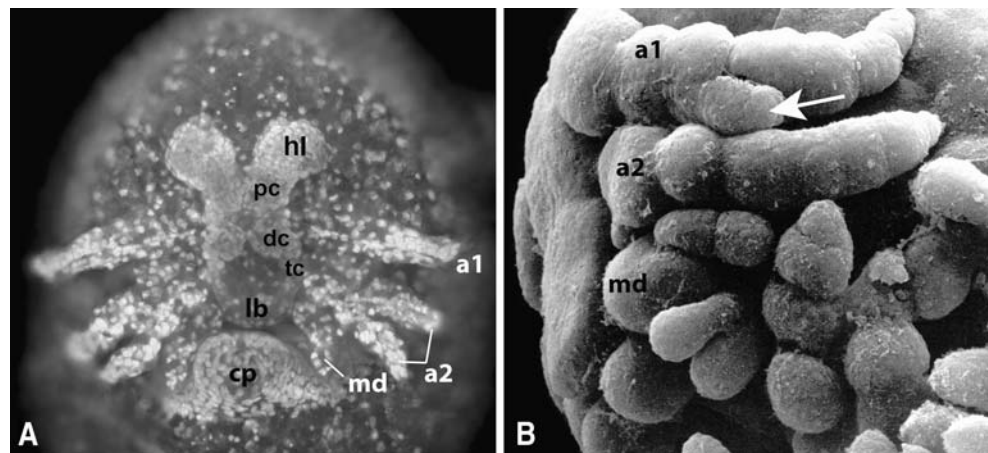
We could not find any of these characters in the remipede naupliar stages we have at hand. Remipede larvae are characterized by the occurrence of numerous small cells with an irregular arrangement. Moreover, there are no teloblasts in the growth zone (at least not in the ectoderm), and a recognizable midline is only found relatively late in development during gangliogenesis. A ventrally folded caudal papilla is not present in the stages examined; the larval trunk of

remipedes remains straight throughout development. The limb buds originate on the ventral side and grow out more or less at a right angle to the ventral surface.

Among malacostracans, a proper free-living nauplius larva occurs only in dendrobranchiate Decapoda and in Euphausiacea. Based not only on comparative morphology but also on the phylogenetic position within the Malacostraca (e.g., Richter and Scholtz 2001), it has been suggested that these malacostracan nauplii are secondarily free-living larvae derived from an embryonic “egg nauplius,” which in turn implies that they are not homologous to nauplii of other crustacean taxa (Scholtz 2000). Nevertheless, some similarities between malacostracan nauplii and those of remipedes can be stated (see also Koenemann et al. 2007a). These concern the lecithotrophic character of the larvae containing a large amount of yolk and lacking mouth, anus, and gut. In addition, the second antennae and mandibles of early naupliar stages are devoid of gnathobasic structures, and the labrum occurs only late in development. However, these correspondences are not enough to claim homology, since most of these features are characteristic for lecithotrophic nauplii in subgroups of various crustacean taxa such as Cirripedia, Branchiopoda, and Copepoda (Dahms 1989; Walossek et al. 1996; Olesen and Grygier 2004).

However, there are several additional correspondences between the remipede nauplii and early malacostracan embryos. These concern, for example, the early developmental pattern of the nervous system in the naupliar region. The cell condensations in the area of the protocerebrum, the deutocerebrum, and the tritocerebrum show a similar specific form, spatial arrangement with respect to the corresponding appendages, and overall appearance between remipede and malacostracan larvae and embryos (Figs. 2c, 3c, and 8a). In particular, the early differentiation of the deutocerebrum as distinct round anlagen finds no counterpart in branchiopods, cirripedes, or copepods (unpublished

Fig. 8 Comparison with Malacostraca. **a** Ventral view of a fluorescent nuclei staining of an egg nauplius of *Anaspides tasmaniae*. **b** SEM photo of the left head region of *Gammarus pulex* with the first and second antenna (*a1*, *a2*) and the mandible (*md*). The white arrow points to a small lateral outgrowth at the first antenna. *a1* first antenna, *a2* second antennae, *cp* caudal papilla, *hl* head lobe, *lb* labrum, *md* mandible, *dc* deutocerebrum anlage, *pc* protocerebrum anlage, *tc* tritocerebrum anlage



data). Surprisingly, the protocerebrum features very distinct head lobes that usually give rise to the lateral protocerebral structures and the compound eyes in Crustacea (see Figs. 2, 3, and 8). Although compound eyes are absent in Remipedia, Fanenbruck et al. (2004) and Fanenbruck and Harzsch (2005) have shown that the organization of the lateral protocerebrum of remipedes is astonishingly complex, comprising a medulla terminalis and a hemiellipsoid body. This complexity might be related to the presence of frontal filaments, which are innervated by the lateral protocerebrum and which are thought to function as sensory organs (see Fanenbruck and Harzsch 2005).

The second correspondence is related to the morphogenesis and differentiation of the first antennae. In both groups, we find first antennae with two branches (Gruner 1993; Boxshall 2004). However, the first antennae of malacostracans and remipedes are controversially interpreted. Some authors suggest that the remipede condition is a true crustacean biramous limb, with the two branches forming an exopod and an endopod, whereas the first antenna of malacostracans is considered to be composed of a main flagellum combined with a minor lateral flagellum (*Nebengeißel*; Gruner 1993). In contrast to this, Boxshall (2004) has shown that, in remipedes, the dorsal branch of the first antenna is segmented and equipped with intrinsic musculature, while the ventral branch is an annulated flagellum lacking muscles. Accordingly, Boxshall (2004) suggests that the first antenna in Remipedia can be regarded as a uniramous limb carrying a secondary ventral flagellum, opposed to two flagella in malacostracans. A comparison between the early differentiation of the first antennae of remipedes and malacostracans reveals that this process is quite similar in the two groups. In either case, there is a long branch constituting the main axis of the limb and a lateral outgrowth that appears relatively late, forming a minor side branch. The main branch appears segmented in the later larval stages of remipedes, and this is also true in the early stages of malacostracan representatives. For instance, the embryos of the amphipod *Gammarus pulex* clearly exhibit the anlagen of segments, indicating that the annulated flagellum is a secondary development of more advanced stages (Scholtz 1990) (Fig. 8). This demonstrates that the general developmental pattern of the first antenna is similar in remipedes and malacostracans and that the two-branched first antenna in both groups is different in development and differentiation from the biramous limbs in the posterior head and trunk regions as proposed for the crustacean ground pattern (see Hejnol and Scholtz 2004; Wolff and Scholtz 2008). In these biramous limbs, the two major rami, exopodite, and endopodite appear early and more or less simultaneously during development (Hejnol and Scholtz 2004; Wolff and Scholtz 2008), as exemplified in the trunk of larval remipedes (Koenemann et al. 2007a; this study). In any case, a first antenna with two branches in

Remipedia and Malacostraca stands in contrast to all other crustacean taxa (including both crown group and stem lineages) that are characterized by a plesiomorphically uniramous first antenna.

A third similarity between Remipedia and Malacostraca concerns the shape of the exopod of the second antennae. Malacostracans deviate from the plesiomorphic crustacean pattern of biramous antennae with multisegmented exopods in that they apomorphically possess second antennae with an unsegmented, scale-like exopod (the scaphocerite; Richter and Scholtz 2001). The transformation of a segmented elongated exopod into the scale-like structure of adults has been described in several malacostracan embryos and larvae (Motoh 1981; Sars 1898; Maas and Waloszek 2001; Alwes and Scholtz 2006). The lack of antennal segmentation of the exopod in adults and its transformation into a scale-like structure during development (Fig. 5d) is also seen in remipedes (Schram 1986, this study).

Larval and developmental similarities to other Crustacea

Some aspects of morphogenesis and cellular characteristics of the naupliar stages in Remipedia resemble those of branchiopod larvae. These include the straight elongated larval shape, a high number of irregularly arranged cells, and the late midline formation (Dohle et al. 2004). However, these are features that are probably plesiomorphic among crustaceans or euarthropods in general (Scholtz 1997). Apart from these shared plesiomorphies, limb formation is entirely different between remipedes and branchiopods.

A general similarity between Remipedia and some other crustaceans, such as Branchiopoda, Cephalocarida, and part of the Malacostraca, is the lack of a mandibular palp in adults. The presence of branched mandibles is considered plesiomorphic in crustaceans, and the retention of only a coxa with a gnathal process would therefore represent an apomorphy. The mandibular rami are retained as probably non-functional vestiges in the pre-juvenile specimen of the Remipedia (Figs. 5a, c), but there is no distinguished similarity to the palp rudiments seen in late branchiopod larvae (Olesen 2004). Since the loss of the mandibular palp has occurred so often in several crustacean lineages, not to speak of hexapods and myriapods (Scholtz et al. 1998), we consider this character as being of little value to resolve remipede affinities.

The strange type of naupliar appendages, in particular the second antennae and mandibles, with no proper articulation but an annulation allowing for a very high flexibility finds no correspondence among other crustacean nauplii, recent or fossil. Interestingly, the “Orsten” fossil “larva C”, an unassignable Paleozoic arthropod larva, is equipped with similar second antennae and mandibles (Müller and Walossek 1986). However, since the affinities between “larva C” and

recent crustaceans are not clear (Müller and Walossek 1986), the corresponding naupliar limbs between Remipedia and “larva C” may be a convergent feature.

In summary, the larvae of remipedes show many characters that are plesiomorphic among crustaceans. Unfortunately, as with other morphological characters of the Remipedia, the larval stages do not present clear apomorphic characters that are shared with another crustacean monophylum. Nevertheless, brain development, the formation and differentiation of the first antennae, as well as the non-segmented second antennal exopod resemble those of malacostracans, supporting the previously suggested close relationship between these two crustacean taxa (Fanenbruck et al. 2004).

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