ORIGINAL ARTICLE

Anatomy of the Tantulocarida: first results obtained using TEM and CLSM. Part I: tantulus larva

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Abstract

The morphology of Tantulocarida, a group of minutely sized ectoparasitic Crustacea, is described here using for the first time transmission electron microscopy (TEM) and confocal laser scanning microscopy (CLSM). This enabled a detailed analysis of their internal anatomy to a level of detail not possible with previous light microscopic investigations. We studied the infective stage, the tantulus larva, attached on the crustacean host in two species, *Arcticotantulus pertzovi* and *Microdajus tchesunovi*, and put special emphasis on cuticular structures, muscles, adhesive glands used in attachment, sensory and nervous systems, and on the organs used in obtaining nutrients from the host. This allowed description of structures that have remained enigmatic or unknown until now. A doubly folded cuticular attachment disc is located at the anterior-ventral part of the cephalon and used for gluing the larva to the host surface with a cement substance released under the disc. Four cuticular canals run from a cement gland, located ventrally in the cephalon, and enter an unpaired, cuticular proboscis. The proboscis can be protruded outside through a separate opening above the mouth and is used for releasing cement. An unpaired and anteriorly completely solid cuticular stylet is located centrally in the cephalon and is used only for making a 1-μm-diameter hole in the host cuticle, through which passes a rootlet system used for obtaining nutrients. The rootlet system is a direct extension of the anterior gut of the tantulus, but inside the host, it consists of cuticle only. Several muscular systems seem to degenerate very quickly after the tantulus has settled on the host. The central nervous system is reduced to an absolute minimum in terms of both cell size and number. We discuss the morphology of the tantulus in light of the Tantulocarida being a sister group to the Thecostraca or even nested within that taxon.

Keywords Tantulocarida · Thecostraca · Comparative anatomy · Phylogeny · TEM · CLSM

Introduction

The Tantulocarida is a group of minutely sized and ectoparasitic Crustacea, infesting other meiobenthic crustaceans and mostly in the deep sea (Copepoda, Isopoda, Amphipoda, Ostracoda, Cumacea, Tanaidacea). Described as a class only in 1983, tantulocarids have a truly enigmatic morphology and remain one of the most poorly studied groups of arthropods. A tentative life cycle of the Tantulocarida has been reconstructed based on data gleaned from several different species (Huys et al. 1993), but they have never been successfully reared in the laboratory. The infective larval stage called the tantulus is one of the smallest multicellular organisms, but nevertheless it retains a general crustacean body plan (Olesen et al. 2014). This minute stage consists of a cephalon, six thoracic somites with paired natatory thoracopods, one limbless somite, and an unsegmented abdomen (Figs. 1c and 2). The cephalon lacks any appendages, but has an oval-shaped, sucker-like attachment organ located on its ventral side (Fig. 3) and also possesses a cuticular stylet for penetrating the cuticle of the host.

In the currently accepted life cycle, the free-swimming tantulus eventually settles on the prospective host (Fig. 1), pierces the host cuticle, and starts feeding from it by means

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Fig. 1 General morphology and rootlet system of the Tantulocarida (CLSM). Parthenogenetic female (**a**, **b**) and tantulus larva (**c**) of *Arcticotantulus pertzovi* on copepod host *Bradya typica*. **a** Parthenogenetic female attached to the host cuticle, general view, dorsally. **b** Cephalon and anterior part of the egg sac, a rootlet system originating from under the oral disc of the parasite. **c** Tantulus larva attached to the host cuticle with rootlet system at early stage of development. r.s., rootlet system; st., stylet; u.c., umbilical cord. Scale bars in micrometers
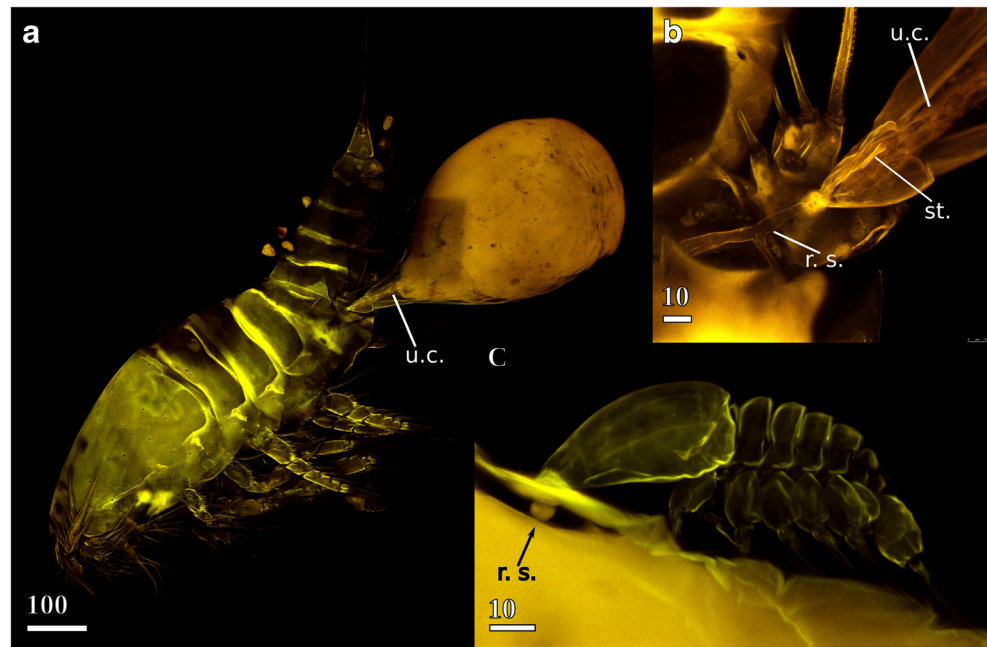
of the rootlet system that enters through the hole (Fig. 1b, c). The attached tantulus now passes through a complex metamorphosis leading to either a parthenogenetic female or to sexual male or female forms. The parthenogenetic female is a sac-like stage that remains attached to the tantulus and thus the host. It contains numerous eggs from which develop a new generation of infective tantulus larvae (Fig. 1a). The second type of metamorphosis results in a single male or female sexual stages. These form from the attached tantulus, but are subsequently are released as free-living, non-feeding males and females equipped with natatory thoracopods. After a presumably internal fertilization, the free female releases a new generation of tantulus larvae. While already by itself complex, this hypothetical life cycle (Huys et al. 1993) is also challenged by the observation of a very aberrant nauplius stage that does not fit into the currently accepted scheme (P.

Fig. 2 General morphology of the tantulus larva. **a** *Serratotantulus chetoprudae* (SEM). **b** *Arcticotantulus pertzovi* (TEM, longitudinal section)
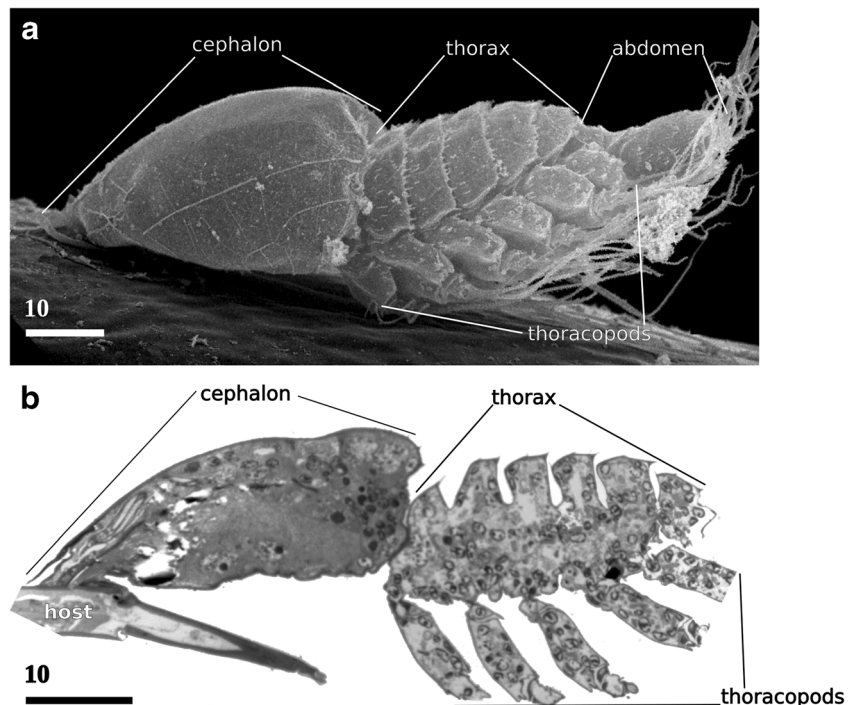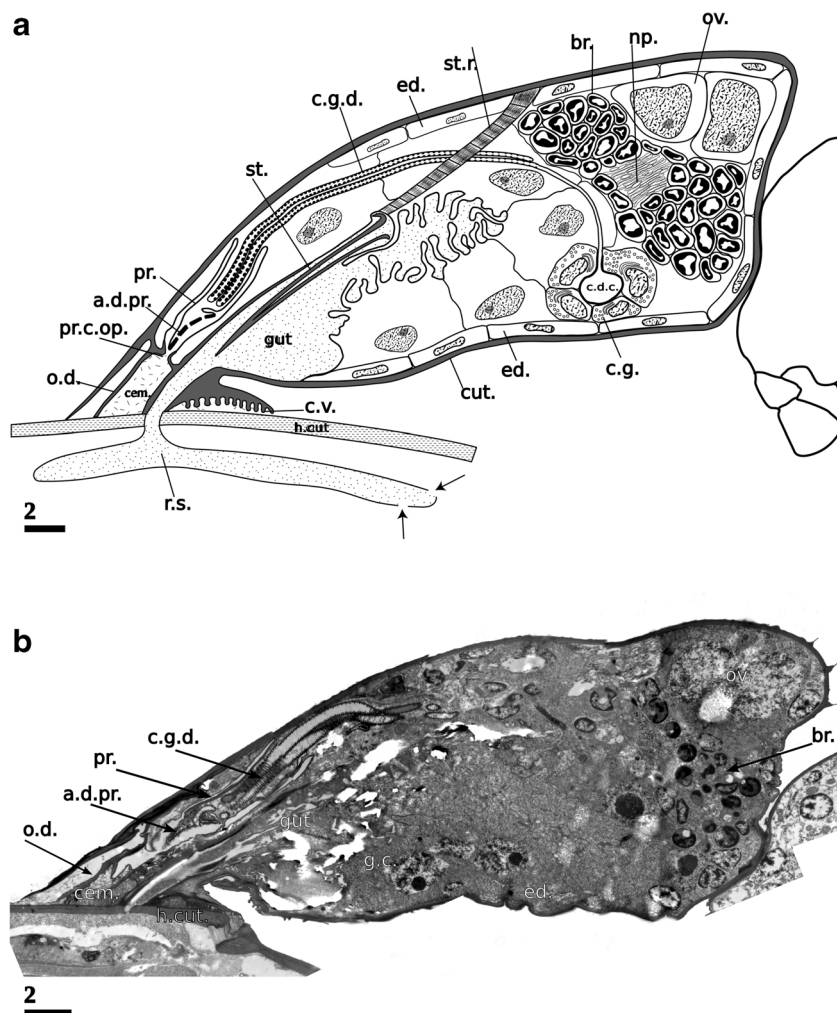
Fig. 3 Cephalon of the tantulus larva: general anatomy on longitudinal cross section. **a** Schematic drawing, nutrition holes in cuticle of rootlet system indicated by arrows. **b** TEM (*Arcticotantulus pertzovi*). a.d.pr., anterior disc of proboscis; br., brain; cem., cement; c.g., cement gland; c.g.c., cement gland cavity; c.g.d., cement gland duct; cut., cuticle; c.v., cuticular villi of the oral disc; ed., epidermis; g.c., gut cell; h.cut., host cuticle; np., neuropil; o.d., oral disc; ov., ovary/germinative cells; pr., proboscis; pr. c. op., proboscis cavity opening; r.s., rootlet system; st., stylet; st.r., stylet retractor. Scale bars in micrometers

two other groups of Thecostraca are also either obligatory parasites (the Ascothoracida) or assumed to be so (the Facetotecta), and the facetotectans even have a metamorphosis, which rivals the rhizocephalans in complexity (Glenner et al. 2008). Obviously, therefore, it is of high interest to study the tantulus and compare it in detail with the infective stages and general adaptation to parasitism in other Thecostraca. But unlike rhizocephalans, where the settlement and host infection has been studied in minute detail with transmission electron microscopy, the tantulus and all other stages of the tantulocarid life cycle has until now been examined by light microscopy only. These studies have revealed a lot of structural details, but they also raise many questions and are in general very difficult to compare with TEM level information. A very preliminary TEM study of the tantulus was given by Petrunina et al. (2014). The purpose of this paper is therefore to provide a comprehensive description of the anatomy of the tantulus larva using both TEM and CLSM techniques. Based on this, we will compare the results with previous light microscopic accounts and thereby provide a platform for discussing tantulocarid relationships and evolution.

Martinez Arbizu, personal communication). It is therefore apparent that we still lack a satisfactory understanding of the life cycle for tantulocarids.

The tantulus fulfills the same role as the cypridoid larva in the hypothetically closely related Thecostraca by being responsible for attachment and metamorphosis into the ensuing stages. Furthermore, like the kentrogon stage in parasitic rhizocephalan cirripedes, the tantulus possesses a cuticular stylet, passes through a complex metamorphosis, and the resulting parasite derives nutrients by means of a system of rootlets inside the crustacean host. On the other hand, the rhizocephalan parasite is first injected as an entirely endoparasitic stage that only later emerges on the surface of the host, while the tantulocarid parasite remains external except for the rootlets.

The apparently close relationship between the Tantulocarida and the Thecostraca and the similarities between the tantulus and the rhizocephalan kentrogon obviously raise the question whether the two groups could be closely related or whether tantulocarid parasitism evolved convergently. It adds to the complexity of this problem that

Materials and methods

Two species of the Tantulocarida *Arcticotantulus pertzovi* Kornev, Tchesunov, Rybnikov, 2004 and *Microdajus tchesunovi* Kolbasov et Savchenko, 2010 are known to parasitize a harpacticoid *Bradya typica* Boeck, 1873 and a tanaid *Typhlotanais* sp. that are common meiobenthic crustaceans inhabiting silty sediments of the Kandalaksha Bay in the vicinity of the White Sea Biological Station (66°31′41″ N, 33°11′08″ E). Bottom mud was obtained from the depths of 20–40 m using light hyperbenthic dredge. A standard bubbling or flotation method was applied to extract meiobenthic crustaceans from the silt (Higgins 1964). Samples were then inspected under a stereo microscope to get tanaids and harpacticoids infested with the Tantulocarida. Specimens at different stages of development attached to their hosts were fixed for electron and confocal microscopy.

For transmission electron microscopy several specimens of settled and metamorphosing tantulus larvae of *A. pertzovi* and *M. tchesunovi* were fixed in 2.5% glutaraldehyde on 0.1 M PBS. The samples were then washed three times for 15 min in PBS and stored at 4 °C before being post-fixed in buffered 1% OsO_4, dehydrated through ethanol series, and embedded in Araldite resin. Ultrathin sections were cut with a diamond knife using Ultracut-R Leica ultratome and stained for 40 min with saturated uranyl acetate and for 5 min with lead citrate. The sections were examined and photographed with a JEOL JEM 100B and JEOl-1011 80 kV.

For scanning electron microscopy, a settled tantulus larva of *Serratotantulus chertoprudae* Savchenko and Kolbasov, 2009 was postfixed in 1% OsO_4, dehydrated in a graded ethanol series and acetone, and critical point dried in CO_2. The specimen was then sputter-coated with platinum–palladium alloy and examined in a JEOL JSM-6380LA microscope at operating voltages of 15–20 kV.

Several specimens of *A. pertzovi* attached to their hosts were fixed with 4% paraformaldehyde in 1 M PBS for 4 h, washed in 1 M PBS, and stained with Congo Red to be observed with TCS CP5 Leica confocal laser scanning microscope.

Photo plates and line drawings were prepared in Inkscape 0.92.

Results

Epidermis and cuticle

The epidermis consists of a 2–3-μm-thick layer of epithelial cells (Figs. 3 and 4) with comparatively large elongated nuclei containing prominent nucleoli (Fig. 5a–f). They also contain endoplasmic reticulum indicative of a high synthetic activity (Fig. 5d). In several specimens, there are multi-membranous bodies of unknown function in the ventral epidermis (Fig. 5b, c). An attachment site for paired muscle strands was observed dorsally in one specimen (Fig. 5e) and by position could possibly have belonged to a stylet retractor (Fig. 3a). The cuticle varies in thickness from 0.20 to 0.70 μm and is clearly differentiated into a thin electron-dense and 20-nm-thick epicuticle and homogenous procuticle (Figs. 5f, g, 3, and 4). Procuticle can consist of up to five irregular layers of different density (Fig. 5g).

Thoracic somites and muscular system

Most of the thorax and the thoracopods are filled with a population of small-sized cells with a high nucleus-to-cytoplasm ratio. The nuclei contain highly condensed chromatin and have almost polygonal shapes with a largest diameter of about ca. 1.5 μm and with very little surrounding cytoplasm. We cannot presently allocate any function to these cells.

Surprisingly, no clear muscles are present in the attached tantulus larvae of *A. pertzovi* (Fig. 6a, c) and *M. tchesunovi* investigated here, except traces of retractor muscles of stylet (Fig. 5e) and degenerated transverse flexor muscles in first thoracic somite (Fig. 6b). In addition, we observed in some specimens the attachment sites of degenerated muscle strands (stylet retractor, thorax and limb flexors), identified by their very characteristic electron-dense semi-desmosome contacts (Figs. 3a and 6b, d).

Oral disc

The attachment apparatus in the form of an oval-shaped (ca. 25 μm long by and 15 μm wide) oral disc is situated anterioventrally on the cephalon (Figs. 3 and 4). The oral disc is partially covered by anterior and lateral cuticular projections, 0.17–0.70 μm thick, while the disc cuticle itself is but 0.13–0.19 μm thick (Fig. 7a, c). The ventral surface is covered with numerous cuticular folds and villi (Fig. 7d–f). It and the whole disc structure is connected to the ventral side of the cephalon by a short cuticular neck, ca. 3.2 μm in diameter and with thick walls enclosing the anterior part of the gut (Fig. 7b, c). Paired cavities (up to 2 μm high) are seen inside the cuticle of the oral disc (Fig. 7c, f), but it is not clear whether they are connected with general body cavity or just represent gaps between cuticle layers. The mouth opening is located centrally on the oral disc (Figs. 3 and 7a, arrowhead) and anteriorly to this a separate opening for the proboscis (Figs. 3a and 4a). In *A. pertzovi*, the entire ventral side of cephalon has numerous longitudinal cuticular ridges composed of both epi- and procuticle (Figs. 5h and 7b).

Cement

Until the system of rootlets grows out into the host, the attachment is ensured entirely by a cement substance presumably of protein nature that is secreted under the oral disc and serves to glue the parasite to the cuticle of the host (Figs. 3, 4, and 7c–f).
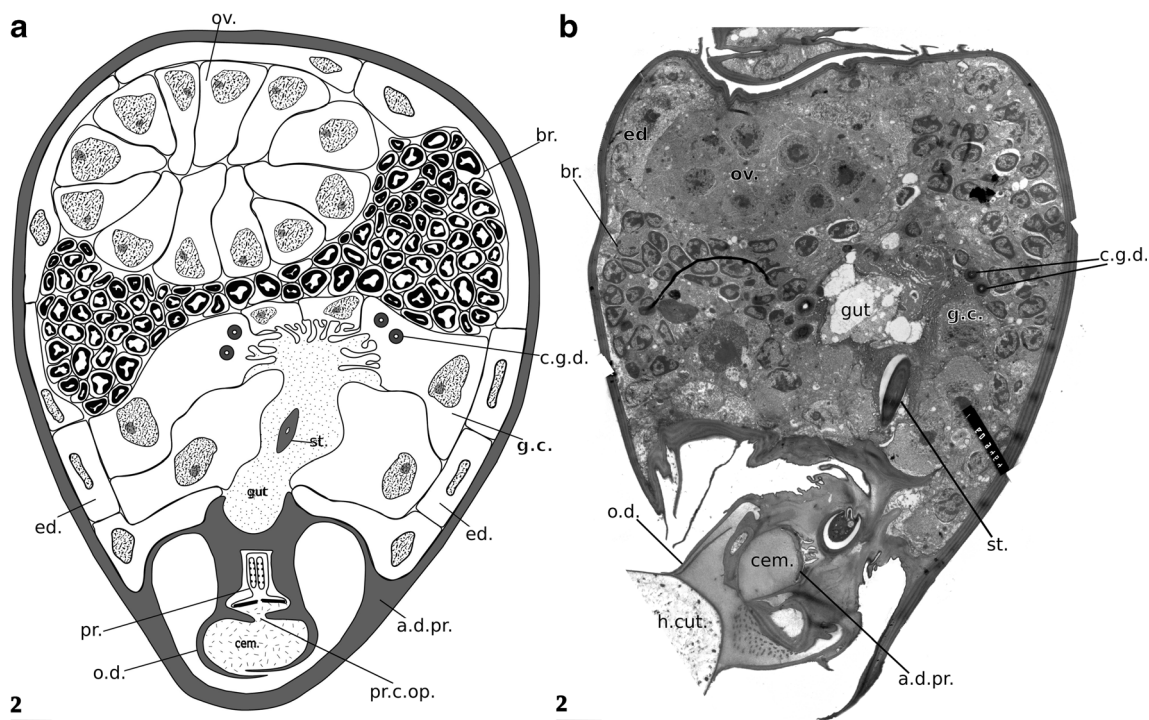
Fig. 4 Cephalon of the tantulus larva: general anatomy on frontal cross-section. **a** Schematic drawing. **b** TEM (*Microdajus tchesunovi*). a.d.pr., anterior disc of proboscis; br., brain; cem., cement; cut., cuticle; c.g.d., cement gland duct; ed., epidermis; g.c., gut cell; o.d., oral disc; ov., ovary/germinative cells; pr., proboscis; pr. cav., proboscis cavity; pr. c. op., proboscis cavity opening; st., stylet. Scale bars in micrometers

The investigated species differed with respect to the type of cement as seen in the TEM pictures. In *A. pertzovi*, the cement is fibrous, consisting of more or less loosely distributed fibers, 15–45 nm thick (Fig. 7d, e). In *M. tchesunovi*, the cement consists of a homogeneous substance of medium electron density (Fig. 7c, f). This difference between the two species was seen both in the cement of newly attached tantuli and in that of already metamorphosed specimens. In *A. pertzovi* and *M. tchesunovi*, the coverage of cement is confined to the space underneath the attachment apparatus (Fig. 7c, d), while in *Serratotantulus chertoprudae*, small portions of the glue appear to be squeezed out (Savchenko and Kolbasov 2009). In detached specimens of *M. tchesunovi*, the cement can be seen to fill the entire volume under the oral disc, having smooth surface that replicates the cuticle of a host (Fig. 7a).

Proboscis

The proboscis is a funnel-shaped, ca. 6-μm-long cuticular structure, situated anteriorly in the cephalon and lying longitudinally above the digestive tract (Figs. 3 and 8a–c, e). Having thin epicuticular walls (Fig. 8e, f), it lies in its own cavity and resembles the finger of a glove intruded into the cephalon (Fig. 8a). The opening of this cavity, observed here for the first time, lies anteriorly to the mouth (Figs. 3a and 4a).

Four cuticular ducts from the cement gland enter the proboscis and terminate with four small pores on its distal part which widens into a discoid shape (Figs. 3, 4, and 8). The structure of cement canals is quite distinct, having very thin walls of epicuticle and are in *A. pertzovi* reinforced by ring-shaped thickenings, rendering them corrugated (Figs. 3 and 8a, b, e, f). The ducts are 0.3–0.5 μm in diameter in the anterior part (Fig. 8e, f), but decreasing slightly toward the posterior end of the cephalon. In *M. tchesunovi*, the duct walls have a smooth 0.8-μm-thick cuticle without any ring structures (Fig. 9a, b).

Cement glands

From the proboscis cement gland, ducts run as a group of four backwards into the dorsal part of the cephalon (Fig. 3). In front of the brain, they split in two pairs and then proceed ventrally (Figs. 4 and 9a), while in *A. pertzovi* also losing the corrugated wall structure. For the major part of their course, there is no epithelium beneath the cuticle of the ducts. Ultimately, the ducts terminate in four large and cuticle-lined cavities located ventrally in the cephalon (Figs. 3 and 9c–e). These cavities have a thicker cuticle than in the ducts proper (Fig. 9d), and they are either spherically shaped (Fig. 9e) or resemble a deflated balloon (Fig. 9c, d). Both the terminal part of the ducts and the terminal cavities lie within what appears to be epithelium cells, and it even seems as if each duct and its particular cavity is associated with one single such cell (Fig. 9b–e) that again forms part of the cement gland (Figs. 3 and 9c, e, f). The ventrally located cement gland consists of secretory cells, having big

Fig. 5 Epidermis and cuticle, tantulus larva (TEM). **a**, **g** *Microdajus tchesunovi*; **b–f**, **h** *Arcticotantulus pertzovi*. **a** Epidermis. **b** Transverse cross-section through cephalon, ventrally. Multimembranous body in epidermis indicated with a star. **c** Longitudinal section through cephalon, multimembranous bodies in epidermis indicated with a star, ventral part. **d** Transverse cross-section through cephalon, epidermal cell with endoplasmic reticulum, ventral part. **e** Transverse cross-section through cephalon showing attachment site of the paired muscle strands to the dorsal body wall. **f** Metamorphosing tantulus larva, transverse section through the middle part of cephalon showing cuticle and epithelium structure. **g** Tantulus larva, sagittal section through the middle of cephalon showing cuticle and epidermis structure. **h** Metamorphosing tantulus larva, ventral margin of cephalon with cuticular ridges, anterior part, transverse section, TEM. ed., epidermis; e.r., endoplasmic reticulum; g., gut; m.a., muscle attachment site; n., nucleus; nu., nucleolus; procut., procuticle; v.r., ventral cuticular ridges. Scale bars in micrometers
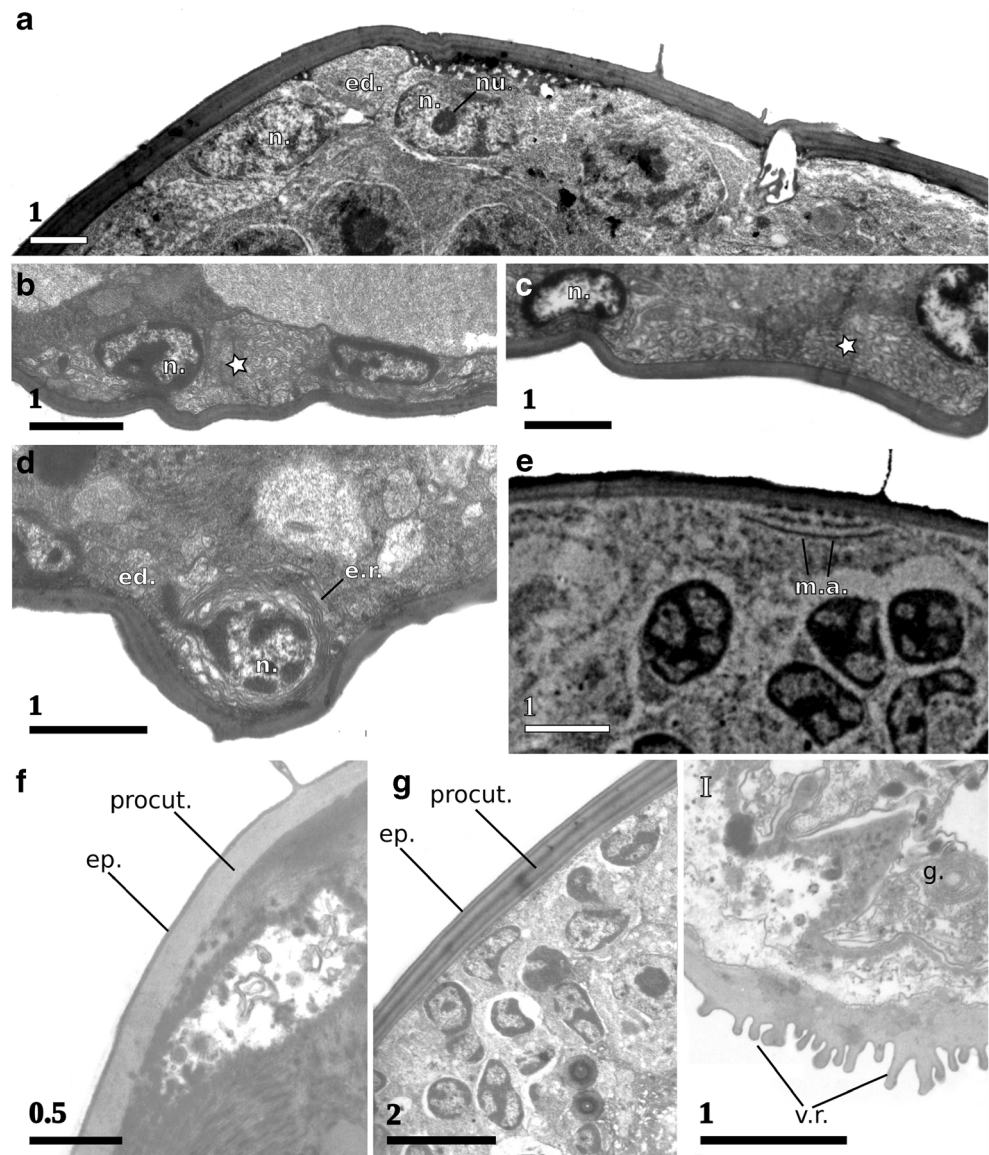
nuclei with distinct nucleoli and a cytoplasm with copious endoplasmic reticulum and as numerous vesicles (Fig. 9c–f).

Stylet

The stylet is an unpaired, cuticular organ located longitudinally in the middle of cephalon (Figs. 1b, 3a, 4, and 10a), where it is already fully developed in the free-swimming tantulus (Huys 1991). In transverse section, it has a roundish shape (Fig. 7b, d) changing to triangular near the proximal end. The diameter is 0.50–0.8 μm at the tip but increasing to ca. 1.8 μm at the basal muscle attachment site (Fig. 10c). It is hollow for most of its length with the cavity opening proximally completely (Fig. 10a–c), but is completely solid at the distal tip (Figs. 3 and 10d, e). The stylet cuticle consists of an outermost 20 nm epicuticle and a procuticle without any distinct fibrous structure and ranging in width from 0.15–0.2 μm

in the distal part to 0.3–0.5 μm in the proximal part (Fig. 10b–d). In *M. tchesunovi*, the basal part of the stylet expands dorsally and curves forward ventrally (Fig. 10a).

Rootlet system and gut

The free-swimming tantulus larva is a non-feeding stage, but soon after settlement it turns into an ectoparasite that feeds by means of a system of rootlets inside the host tissues. Newly attached tantuli have no rootlets. We could not observe the rootlet formation directly, but in attached tantuli assumed to be slightly older, the anterior and cuticular part of the gut has sent an outgrowth into the host through the hole produced by the stylet (Figs. 1b, c and 3). The form of the resulting rootlet system is variable, but seems normally to consist of main trunk that can issue branches, perhaps depending on the age of the parasite (Figs. 1b and 11). The walls of the rootlet consists of a

Fig. 6 Internal structure of the thoracic segments, tantulus larva, TEM (*Arcticotantulus pertzovi*). **a**, **c** Longitudinal cross-sections through whole body and first and second thoracic segments. **b**, **d** Transverse cross-sections through first thoracic segment with traces of dorsoventral muscles and muscle attachment sites, ventral part enlarged in (**d**). ceph., cephalon; d. m., disintegrating muscles; m.a., muscle attachment site; thp., thoracopods. Scale bars in micrometers
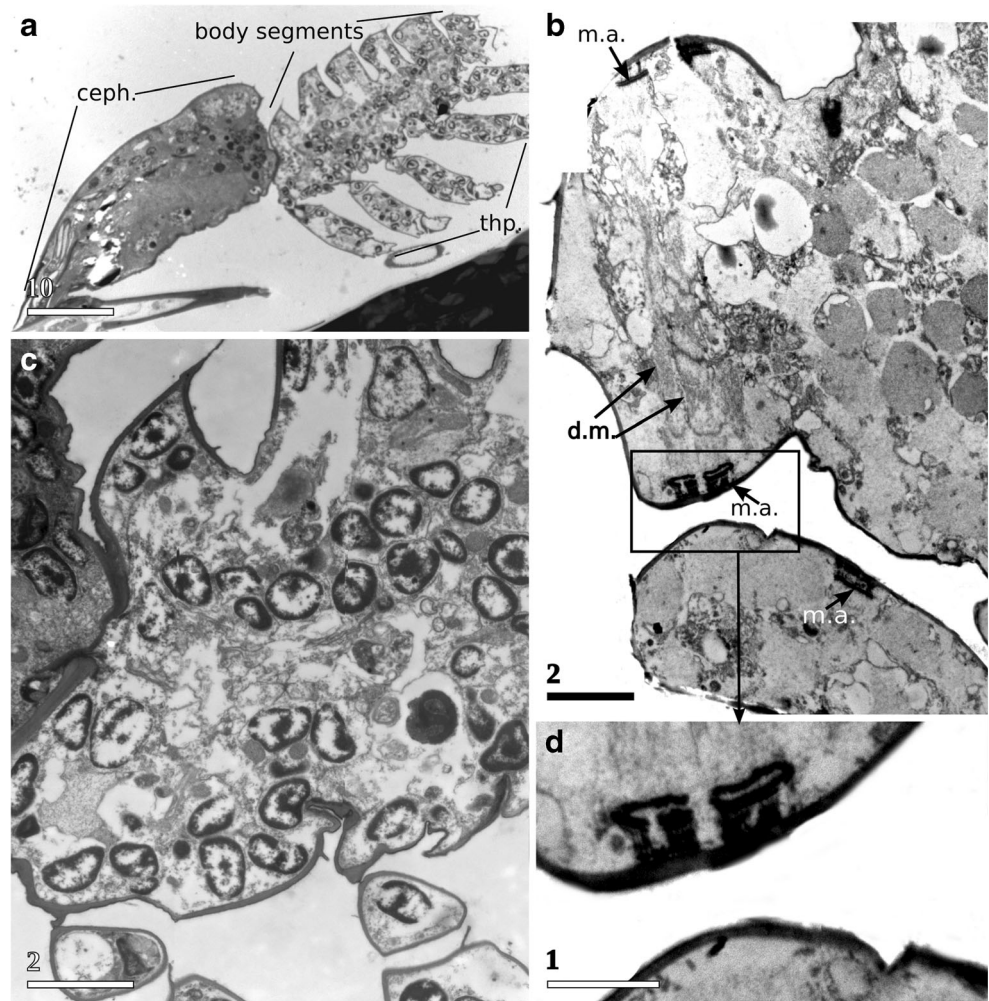
very thin cuticle (60–140 nm), in which both a procuticle and an epicuticle can nevertheless be distinguished (Fig. 11b, c, e). Surprisingly, there seems to be no underlying epidermis or any other cellular elements inside the rootlets, which therefore appears to be entirely cuticular. Newly attached tantuli have not yet developed the rootlet system, but could nevertheless have material inside their gut cavity, indicating they can absorb nutrients through the mouth opening which is oppressed to the puncture made by stylet (Figs. 11d and 12f, g).

At the mouth opening in the central part of the oral disc, the rootlet system is directly continuous with the anteriormost and entirely cuticular part of the gut (Fig. 11a). From the mouth, the gut then continues inside a cuticular thickening of the oral disc (neck region) (Figs. 3 and 12c, f). Further inside the cephalon, the gut first retains a thick cuticular wall (Fig. 12a), but this is eventually replaced by very thin epicuticle (Figs. 3, 4, and 12b, c, f). In this region, the gut cuticle lies above an epithelium consisting of very large (4–6 μm) cells with big nuclei (about 2 μm) and containing fibrinous chromatin evenly spread inside a round-shaped nucleus (Figs. 3, 4, and 12c, d, f). The lumen of the gut is only distinct in the anterior part, where the wall

consists of cuticle only (Fig. 12a–c, f). Further posteriorly, the gut walls are formed by cells with numerous very thin outgrowths (Figs. 3, 4, and 12e, f) that occupy the entire central space The gut is entirely cephalic since its posterior part adjoins the brain (Fig. 12c, f), and this corresponds with absence of an anus in the tantulus larva. The anterior gut lumen is filled with hemolymph of the host even before a rootlet system has developed inside the host (Figs. 11d and 12e, f).

Brain

The bi-lobed and dumbbell-shaped brain is located in the posterior part of cephalon (Figs. 3, 4, and 13a, c, d), measuring maximally 12 μm in length long, 20 μm in width, and 13 μm in height and with no evident divisions into a proto-, deuto-, and tritocerebrum. The particular form and location of the brain depends on both the species and the future development pathway taken by the larva. In a tantulus destined to metamorphose into a parthenogenetic female, the ovary develops in the posterior part of the cephalon (Figs. 3, 4, and 13c), thus shifting the brain slightly toward the anterior part. The total
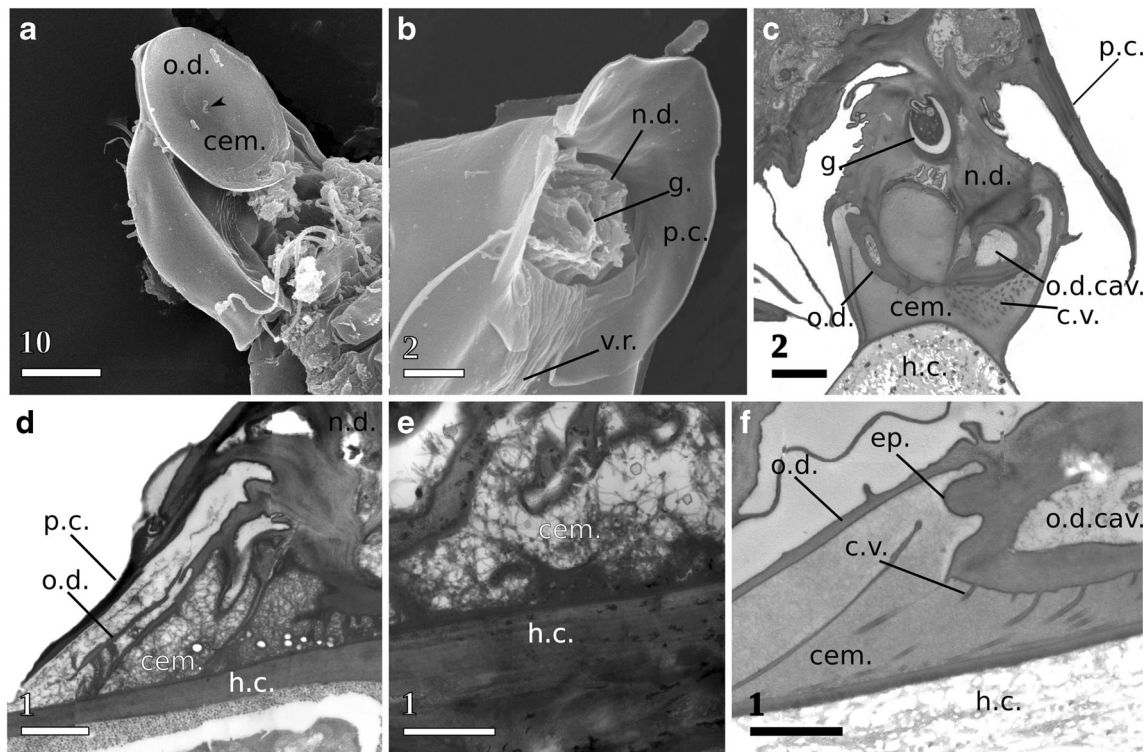
Fig. 7 Attachment apparatus of the tantulus larva (**a**, **c**, **e** *Microdajus tchesunovi*; **b**, **d**, **e** *Arcticotantulus pertzovi*). **a** Metamorphosing tantulus larva. Ventral side of the cephalon with oral disc covered with cement, mouth opening on the disc marked with an arrowhead, SEM. **b** Metamorphosing tantulus larva, anterior part of cephalon, oral disc removed showing cuticular neck of the oral disc with the gut centrally, SEM. **c** Newly attached tantulus larva, transverse section of the anterior part of cephalon, TEM. **d** Tantulus larva, anteriormost part of cephalon, longitudinal section, TEM. **e** Transverse section of the oral disc showing cement structure, TEM. **f** Transverse section of the anteriormost part of cephalon with oral disc showing cuticular villi, TEM. cem., cement; c. v., cuticular villi of the oral disc; ep., epicuticle; g., gut; h.c., host cuticle; n.d., neck of the oral disc; o.d., oral disc; o.d.cav., cavity in oral disc; p.c., projections of cephalon. Scale bars in micrometers

number of pericarya is estimated to be approximately 220–250. They are very small (slightly more than 1 µm) and lie close together as a cortical layer surrounding distinct, ca. 4-µm-wide neuropil. The cortex of cell bodies is 6–7 cells thick in the mid-dorsal part and 2–3 cells thick laterally and (Fig. 13d, e) with the large ca. 1-µm-wide nuclei occupying the major part of the cell volume, leaving only a thin layer of cytoplasm (Fig. 13d, f, g). Masses of highly compact, electron-dense, and homogeneous chromatin are mainly concentrated along the perimeter of the nuclei (Fig. 13f). Electron-dense vesicles indicative of synapses can be seen in the neurite fibers of the neuropil (Fig. 13a, b). There are no clear neuroglial cells adjoining the cell bodies and no distinct enveloping sheath (Fig. 13a, c, d, g).

Sensory structures

The tantulus larva has neither eyes nor sensilla-carrying appendages and the only sensory organs are a number of paired cephalic pores/pits (Fig. 14) with or without sensilla (Fig. 14a, b, g).

Using the pore formula of Boxshall and Vader (1993), *M. tchesunovi* has four pairs of pores distributed on the cephalon according to the formula A_I, D_I, D_{II}, L_I, while in *A. pertzovi*

tantulus larva has 11 pairs of pores (A_I–A_{IV}, D_I–D_{IV}, L_I–L_{III}). In *M. tchesunovi*, the dorsal pores are shifted to the hind margin and the openings of "D" pores face in posterior direction.

Simple pores consist of two parts. An outer pore chamber about 0.6 µm in diameter and 0.5–1 µm deep (Fig. 14e, k–m) located directly in the cuticle and an inner cavity 0.3–0.5 µm wide and 0.5–1 µm deep located in the epidermis (Fig. 14f, k, l). The inner chamber is in contact with a sensory dendritic process. The walls of the pore, both in the outer and inner chambers, are outlined with thin epicuticle, while the constricted and ca. 200-nm-wide communication between the two compartments is reinforced by a neck of thicker cuticle (Fig. 14f, k, l). The sensillate pores/pits lack both cuticular chambers and canal and the dendritic process is directly connected with the minutely sized sensilla (Fig. 14d, e).

Ovary

In tantulus larvae presumed to be metamorphosing into parthenogenetic females, the primordial ovary is located in the rear part of cephalon (Figs. 3, 4, 13c, and 15a). It is a group of large cells forming a compact rosette-like structure and all having the same appearance: (1) high nucleo-cytoplasmic ratio; (2) large

Fig. 8 Proboscis and cement gland ducts, TEM. *Arcticotantulus pertzovi* (**a–c**, **e**, **f**), *Microdajus tchesunovi* (**d**). **a** Tantulus, longitudinal section through the midline of cephalon, anterior part. **b** Tantulus, cement gland ducts inside proboscis, transverse cross-section. **c** Tantulus, anterior disc of proboscis, longitudinal section, openings of the cement gland ducts marked with arrowheads. **d** Cement released through the anterior disc of proboscis under the oral disc, transverse cross-section, opening of the cement gland duct marked with an arrowhead. **e** Metamorphosing parthenogenetic female, cement gland ducts entering proboscis, transverse section through anterior part of cephalon. **f** Tantulus, cement gland ducts with and without cement, longitudinal cross-section. a.d.pr., anterior disc of proboscis; cem., cement; c.g.d., cement gland duct; cut., cuticle; g., gut; h. cut., host cuticle; o.d., oral disc; pr., proboscis; pr. cav., proboscis cavity. Scale bars in micrometers
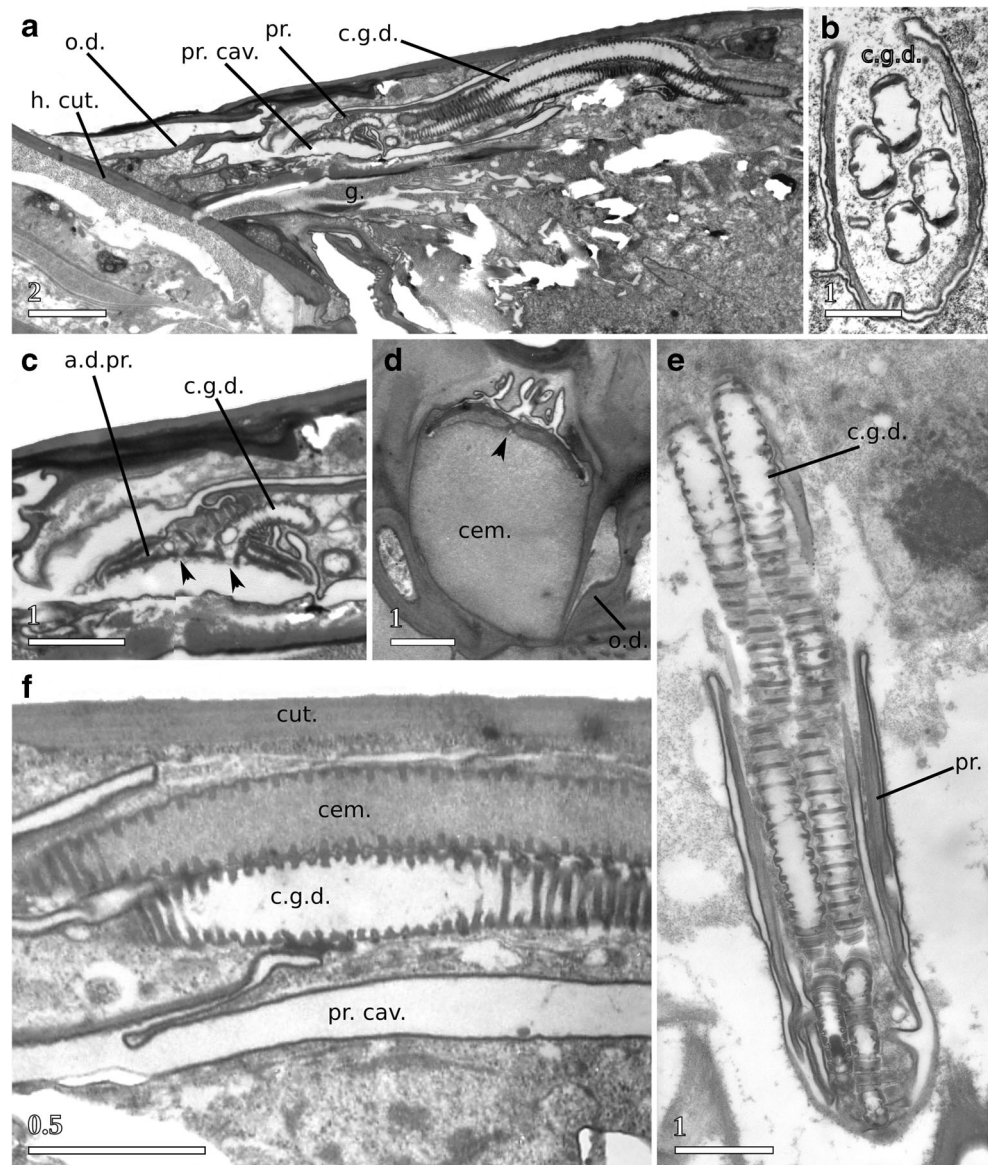
nuclei (about 2.2 μm) with diffuse chromatin; (3) large nucleoli (0.7 μm) (Fig. 10a, b). The cytoplasm contains small, electron-dense areas that may possibly be referred to as nuage (Fig. 15a, b, arrowheads), but we could not find that these dark spots are associated with mitochondria. Since all these characters are typical of germ cells, we consider this organ as the ovary of the parthenogenetic stage, which is then removed from the cephalon to the growing cuticular sac (Fig. 1a).

Discussion

We have for the first time provided a full account of the tantulus larva of the Tantulocarida by means of transmission electron microscopy and supplemented by information using CLSM. This allows us to describe in detail the organ systems of this extremely small stage and correlate with findings with previous studies based on light microscopy or SEM only. With respect to several organ systems, notably the rootlet system and the gut and the cement apparatus, we are for the first time able to provide a comprehensive account that allows both discussion and comparison with other taxa putatively related to the Tantulocarida and their larval stages, most notably the cypris and kentrogon stages of the Cirripedia Rhizocephala.

Attachment apparatus

The attachment process The cephalon of tantulus is devoid of paired appendages, so the proboscis and the associated cement glands are wholly responsible for attachment of the tantulus larva which is ensured entirely by chemical means. Previous SEM studies have shown that the free-swimming tantulus can

Fig. 9 Cement glands, ducts, and cuticular cavities of cement glands (TEM). *Microdajus tchesunovi* (**a**, **b**), tantulus larva, frontal cross-section through central part of cephalon; *Arcticotantulus pertzovi* (**c**–**f**), tantulus larva, transverse cross-section of cephalon through cement glands. **a** Two pairs of cement gland ducts running along the gut. **b** Cement gland ducts. **c** Right part of cement gland (marked with dashed outline) with cuticular cavity and cement gland duct fusing with it. **d** Cuticular cavity of the cement gland. **e** Transverse cross-section of cephalon through cement glands (marked with dashed outline) and cuticular cavities. **f** Transverse cross-section through cement gland. c.g., cement gland; c.g.c., cement gland cavity; c.g.d., cement gland duct; e.r., endoplasmic reticulum; g., gut; n., nucleus; nu., nucleolus. Scale bars in micrometers
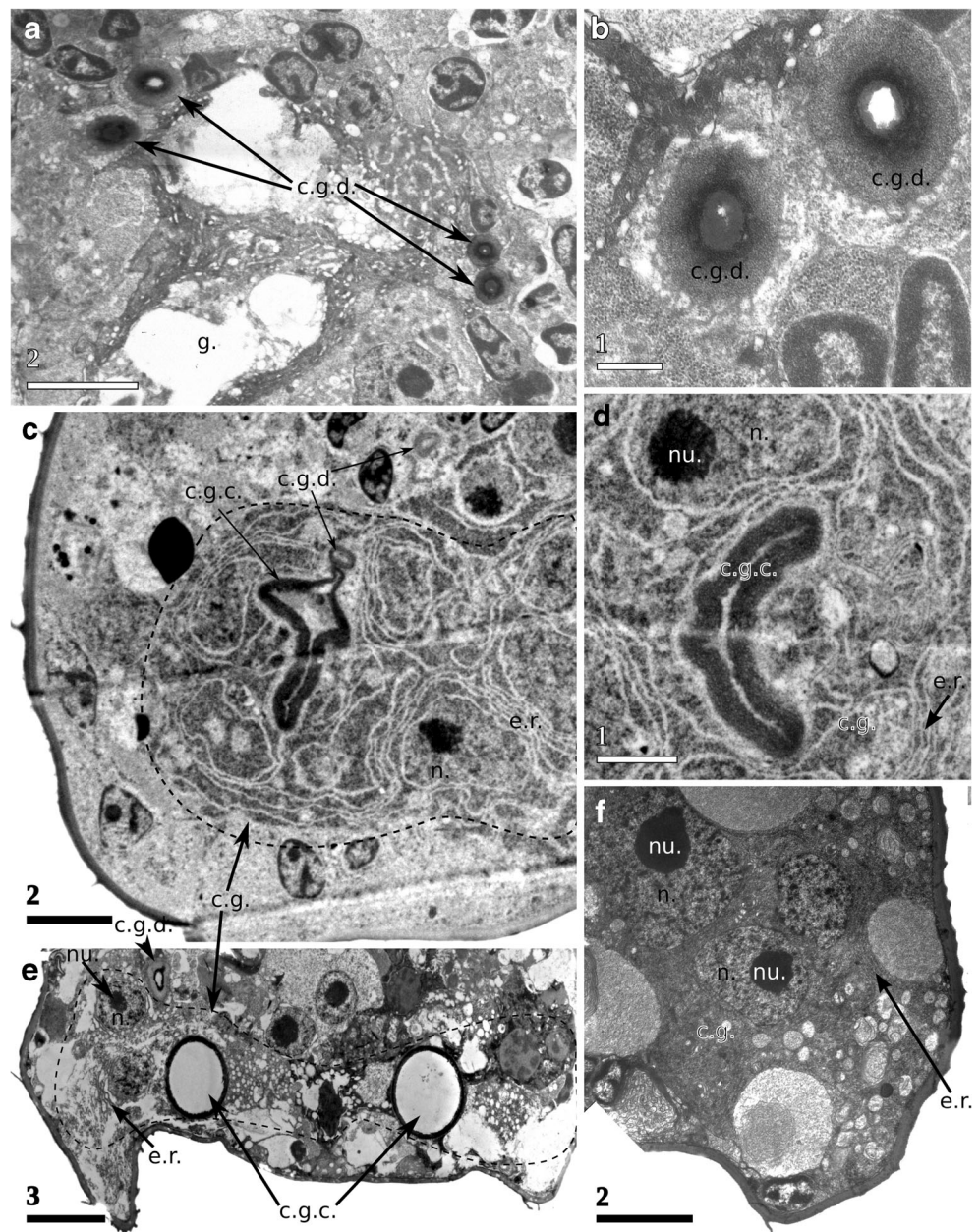
protrude the proboscis out of the cephalon (Huys et al. 1992), but the true position of proboscis cavity and its opening anteriorly to the mouth was observed here for the first time (Figs. 3a and 4a). After initial cement secretion, the proboscis must be withdrawn back into its cavity thus pulling the larva toward the host. This is followed by release of the rest of the cement that can now spread under the entire attachment disc and provide a firm attachment to the surface.

It is somewhat baffling that we could not find any muscles responsible for either the protraction or retraction of the proboscis. This must be explained by a very rapid degradation of myofibers as soon as they have completed their function as suggested by Boxshall (1991). In agreement with this, we did in one specimen find clear muscle attachment sites (Fig. 5e)

that probably were associated with either the proboscis or the stylet retractor muscles. In *Dicrotrichura tricincta* Huys 1989, proboscis was connected with muscular strands running further inside cephalon (Huys 1989), and it was suggested that the so-called striated organ described in other species (Boxshall and Lincoln 1983) is simply a proboscis retractor.

The cement In the tantulus, the cement is secreted only under the oral disc and the adhesion depends upon the chemical properties of this substance. The special morphology of the attachment organ reveals several adaptations to an efficient adhesion. The cuticular outgrowths of the cephalon around the oral disc may well serve to prevent spilling out of cement and the folds and villi on the disc itself will increase the surface area, thus
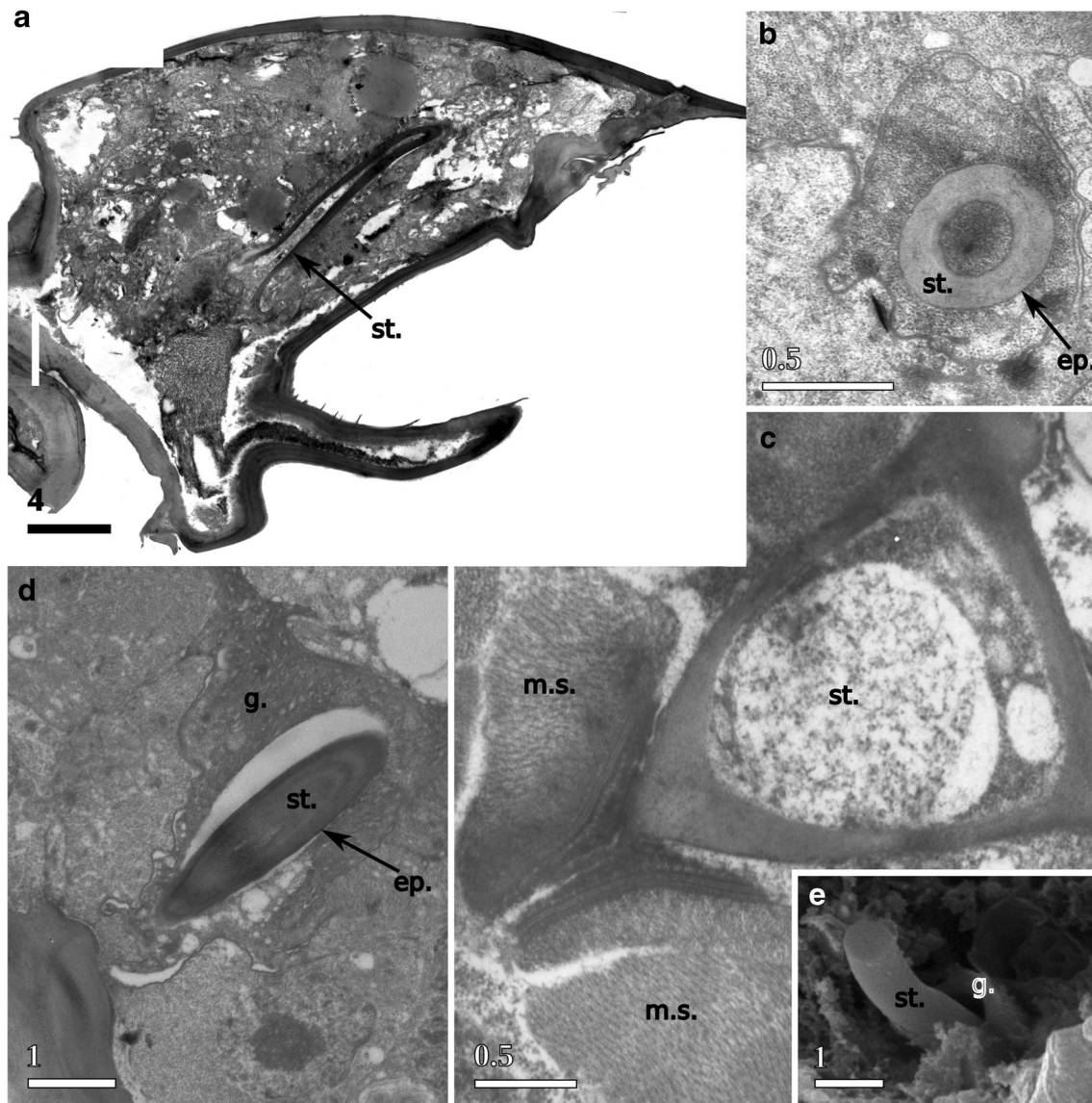
Fig. 10 Stylet of the Tantulocarida (TEM, **a–d**; SEM, **e**). *Microdajus tchesunovi* (**a**, **d**), *Arcticotantulus pertzovi* (**b**, **c**), *Serratotantulus chertoprudae* (**e**). **a** Metamorphosing tantulus, longitudinal section through the midline of cephalon, showing position of stylet. **b** Tantulus, transverse section of stylet, middle part. **c** Tantulus, transverse section of stylet, basal part. **d** Tantulus, sagittal section of stylet, distal part. **e** Solid tip of stylet surrounded by anterior part of gut. ep., epicuticle; g., gut; m.s., muscle strand; st., stylet. Scale bars in micrometers

also serving for firm fixation. Functional similarities to this may again be found in the structure of the antennular attachment organ in cirripede cyprids (Bielecki et al. 2009).

The cement gland The adhesive glue itself is produced in paired cement glands and is transported through four cuticular ducts to the distal end of the proboscis. We suggest that prior to secretion, the cement is stored in the cavities associated with the proximal end of each duct. The actual secretory cells of the gland have all the characteristics of high synthetic activity, and we surmise that the secreted substance is at least in part of a proteinaceous nature. The amount needed for adhesion is very small and released immediately at attachment, so we assume that the cement gland in the settled specimens

studied by us had already started to degrade. Exactly the same is seen in kentrogons of *Lernaeodiscus porcellanae* Muller, 1862 when their cement secretion ceases a few hours after first attachment. Correlation between light microscopy and TEM data is always fraught with difficulty, but we suggest that so-called globular structures and glandular bodies described by several authors from both live and fixed tantuli (Huys 1991; Huys et al. 1992) correspond to the cuticular cavities were here found to be associated with the cement glands.

Digestive system

Stylet The tantulus larva starts feeding only after settlement on host, when it becomes a true parasite. The mechanism of

Fig. 11 Rootlet system of the Tantulocarida (TEM). *Microdajus tchesunovi* (**a**, **e**), *Arcticotantulus pertzovi* (**b**–**d**). **a** Metamorphosing tantulus, transverse section through anterior part of cephalon showing connection of rootlet system with anterior gut. **b** Metamorphosing tantulus, rootlet system inside host tissues, transverse section. **c** Enlarged cuticular wall of the rootlet system. **d** Newly attached tantulus (rootlet system is not developed yet), longitudinal section through anteriormost part of cephalon medially, stylet puncture in host cuticle indicated with arrowhead. **e** Metamorphosing tantulus, rootlet system (filled with host content—hemolymph) in host tissues, transverse section. cem., cement; ep., epicuticle; g., gut; h.c., host cuticle; o.d., oral disc; p.c., projections of cephalon; r.s., rootlet system. Scale bars in micrometers
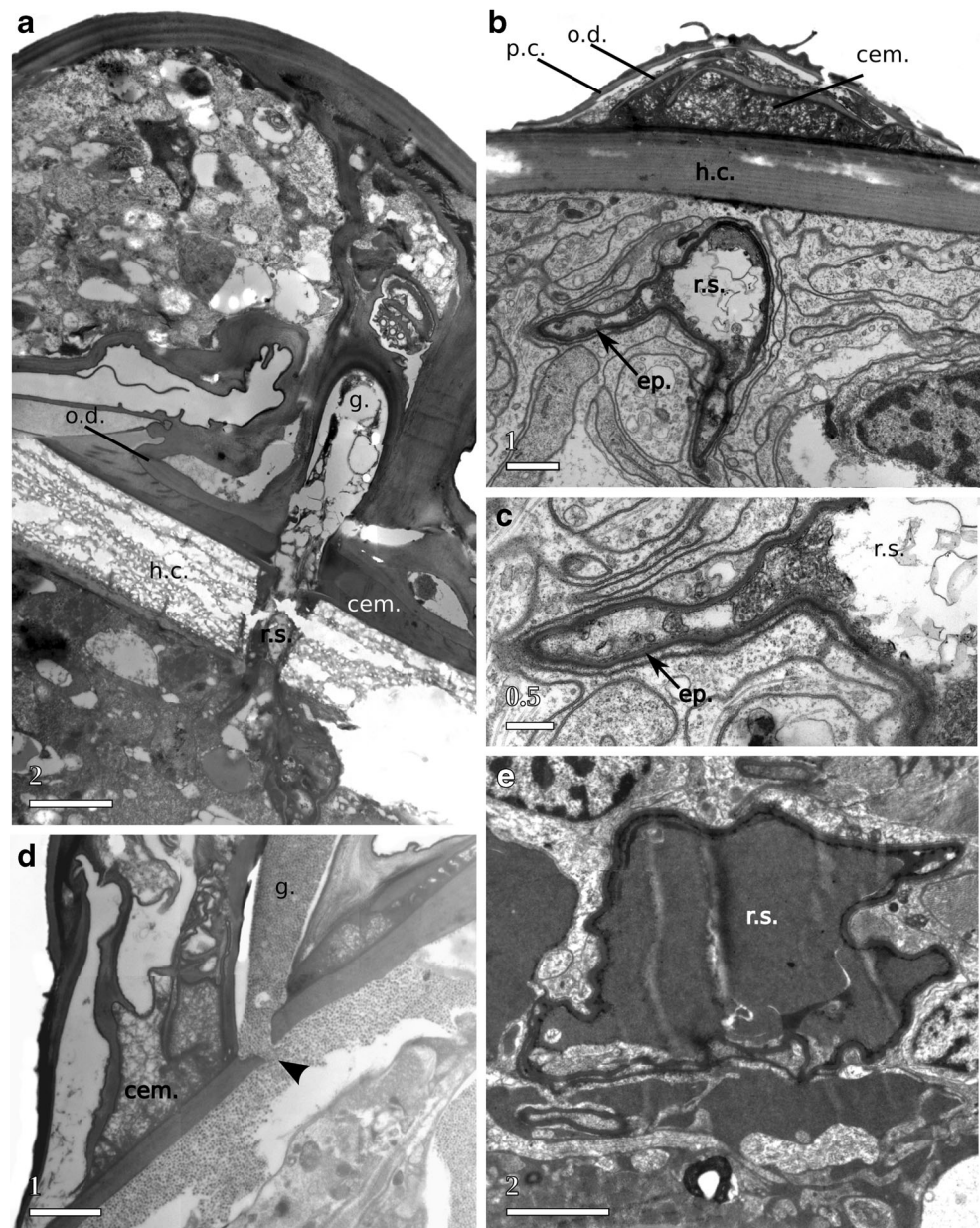

obtaining nutrition was developed "de novo" as an adaptation to the parasitic style of life, and thus it has several unique features. An unpaired cuticular stylet is used to get access to host fluids through a single puncture made by it. The particular mechanism of the stylet movement is not yet fully understood, but protractor and retractor muscles were described in detail in free-swimming larva using light microscopy (Huys 1991; Huys et al. 1992). The protractors lie laterally on both sides of stylet and go from the end of the so-called gubernaculum to the posterior barbs of stylet. Paired retractors originate from the basal part of stylet and are attached dorsally to the cuticle of posterior part of cephalon. We could not observe any of these muscles in our attached specimens, so presumably they start to disintegrate immediately after they have completed their function. Furthermore, we did find

muscular attachment sites that most likely relate to both stylet and proboscis muscles. In *Tantulacus hoegi* Huys, Andersen, Kristensen, 1992, the stylet is claimed to have a thin duct with a distal opening armed with a row of barbs and also to be associated with paired glandular bodies at the basis of stylet, suggested to secrete a substance for dissolving the host cuticle (Huys et al. 1992). In contrast, we found that the stylet always had a solid tip and without any associated glands, so at least for the three species investigated here, it is clear that the stylet is used only for mechanical puncture of host integument.

Rootlet system The opening left by the stylet is so small that the tantulus could not efficiently obtain nutrition from the host without some sort of a pumping mechanism for which we

Fig. 12 Gut anatomy, tantulus larva (TEM). *Arcticotantulus pertzovi* (**a–b**, **e–g**), *Microdajus tchesunovi* (**c**, **d**). **a** Anterior part of cephalon, general view, transverse cross-section showing anterior gut with thick cuticular dorsal wall and distal part of proboscis. **b** Anterior part of cephalon, transverse cross-section, gut with thin cuticular lining (indicated by arrowheads). **c** Frontal cross-section through cephalon, general view of the gut (gut wall indicated by arrowhead) with enlarged part showing the gut cell (**d**). **e–g** Longitudinal cross-section through middle line of cephalon (**f**) with enlarged anteriormost (**g**) and posterior (**e**) parts of gut showing host hemolymph inside the gut. br. c., brain cell; c.g.d., cement gland duct; g., gut; g.c., gut cell; h.c., host cuticle; h.h., host hemolymph; pr., proboscis; st., stylet. Scale bars in micrometers
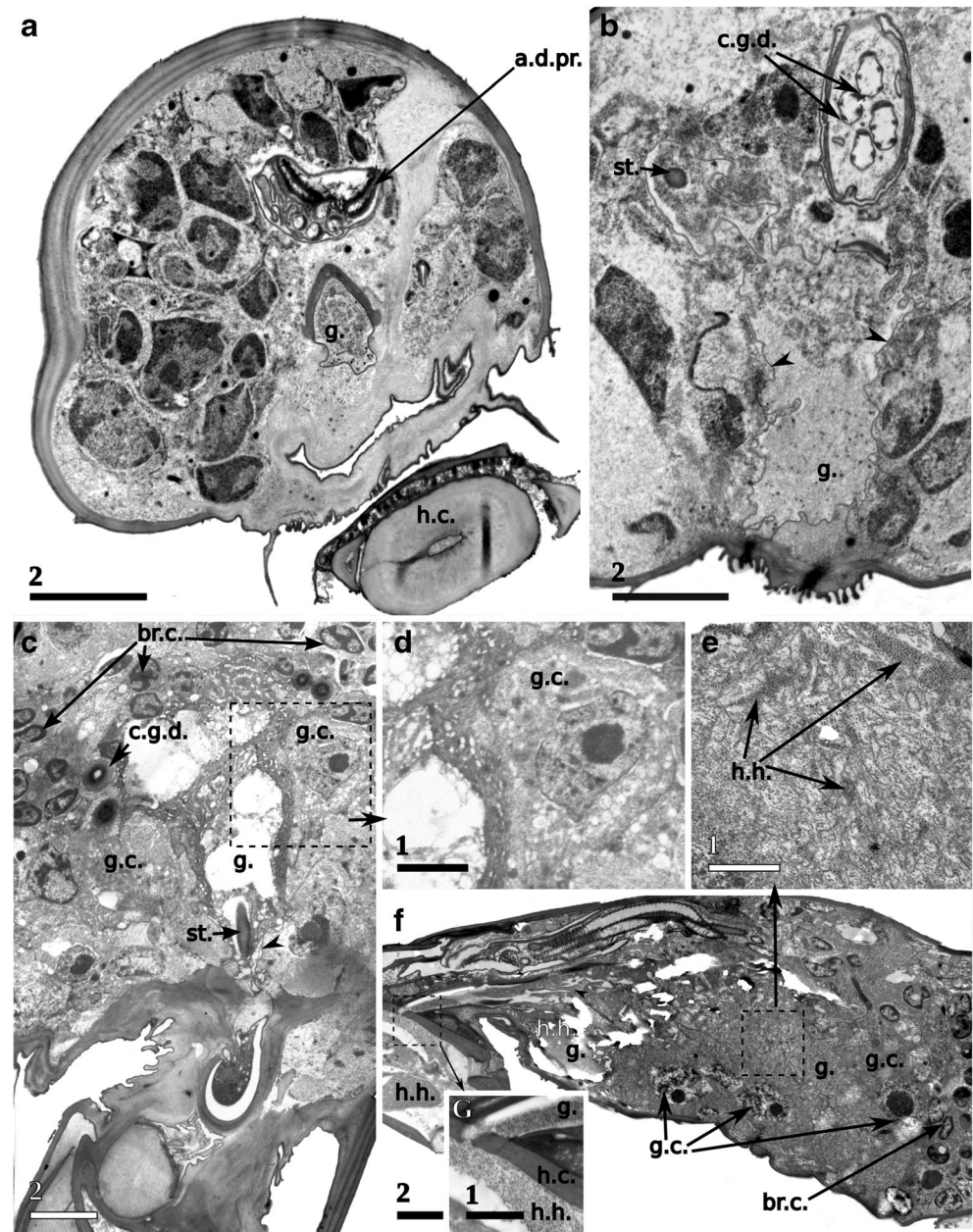

found no evidence. We can now for the first time prove that the rootlet system is in direct communication with the anterior cuticular part of the gut. The rootlets must therefore be the organ by which the parasite feeds from the host, but the details of this function remain obscure. Unlike the well-described rootlet system of rhizocephalan barnacles (Bresciani and Høeg 2001), the rootlet cuticle of tantulocarids is not underlined with any epithelial cells that could function in absorption of nutrients. It is probable that transportation of host juices simply enters the rootlets through tiny holes in the cuticle (Fig. 3, arrows), but this still leaves the question how an efficient flow into the tantulus body is maintained. Possibly, the tantulus is so minute that simple diffusion through the rootlets and into the gut is in fact the active mechanism.

It is not yet evident how the formation of the rootlet system is performed since in our specimens there were no epidermis cells entering the host tissue that could secrete the rootlet cuticle and enable its expansion into a branched system. Although purely speculative, it is possible that such cells are initially present but then withdrawn after cuticle secretion as is known to occur in appendages of some arthropods (Høeg 1985).

The gut The cuticular anterior gut forming a guiding tube for the stylet has been reported for several species (Huys et al. 1992; Savchenko and Kolbasov 2009), but even based on the present TEM data, the structure of the middle gut remains uncertain. A tantulus larva already fixed to host does not have any evident middle gut with a lumen and microvillus-covered

Fig. 13 Brain anatomy, tantulus
larva (TEM). *Arcticotantulus
pertzovi* (**a**, **b**, **d**–**g**), *Microdajus
tchesunovi* (**c**). **a** Longitudinal
cross-section through middle line
of cephalon, brain indicated by
dotted outline. **b** Enlarged part of
brain with electron-dense vesicles
containing mediator (indicated
with arrowhead). **c** Frontal cross-
section through cephalon, brain
indicated by dotted outline. **d**–**g**
Transverse cross-section through
cephalon. **d** Posterior part of brain
with neuropil and bi-lobed cortex
layer. **e** Neuropil, enlarged part of
(**d**). **f** Neurite cell bodies, enlarged
part of (**d**). **g** Anterior part of brain
located dorsally in the cephalon,
transverse cross-section. g., gut;
np., neuropil; ov., ovary; st.,
stylet. Scale bars in micrometers
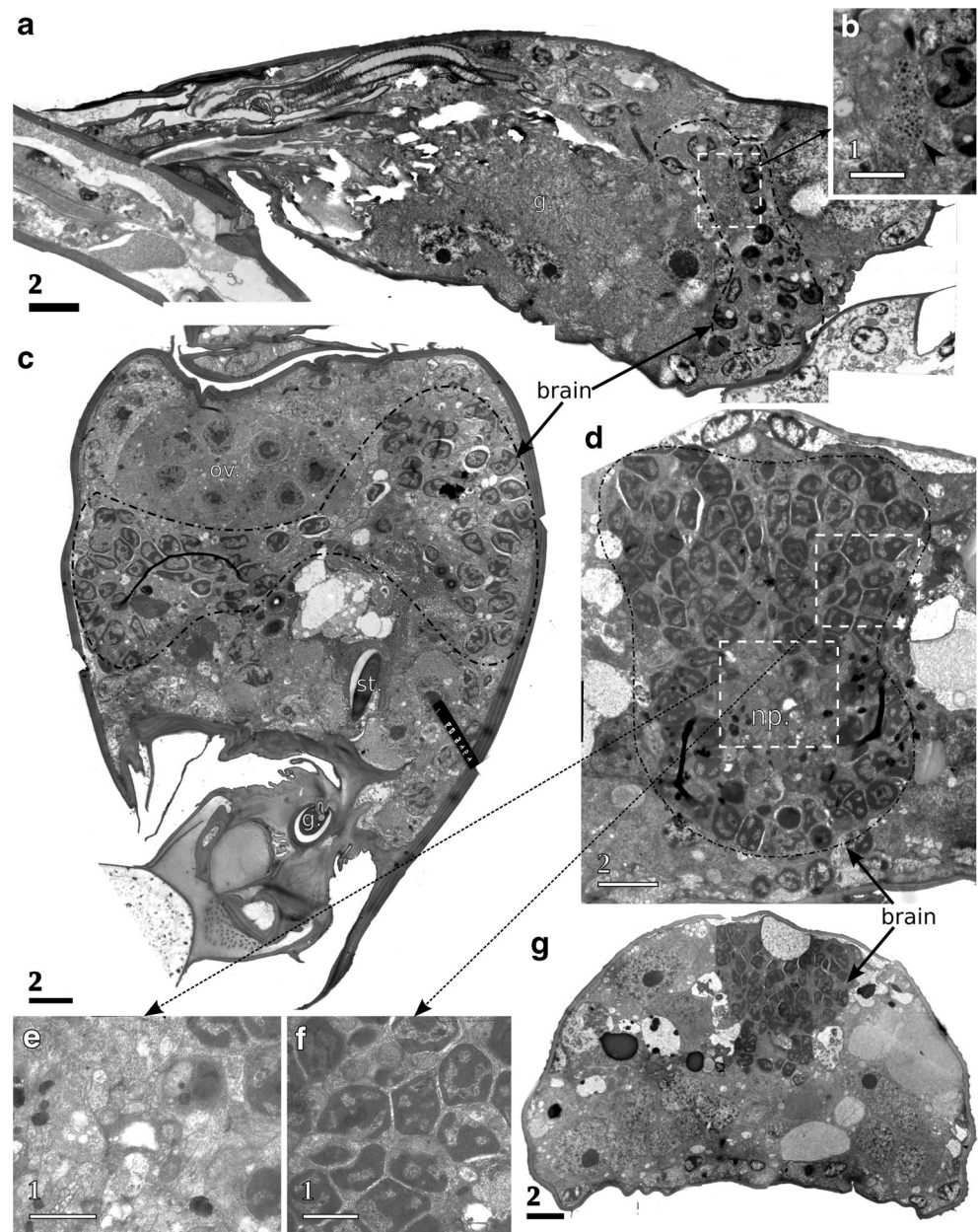
epithelial walls neither inside cephalon (Fig. 12) nor in the
thoracic segments (Fig. 6a–c). Still, the cells here observed
in the posteriormost part of the gut must in some way be the
ones responsible for absorption even if they do not resemble a
traditional gut epithelium.

Additional cuticular structures such as the so called buc-
cal gubernaculum and buccal capsule were described by
Huys (1991) in the free tantulus larvae of *Xenualytus
scotophilus* Huys, 1991 and *Campiloxyphos dineti* Huys,
1990 (Huys 1990). In our study, even in newly attached
tantuli, there was no clearly discernible such gubernaculum
or buccal capsule. The thick cuticle in the anterior part of
the gut serves as guiding tube for the stylet, and this may
well have been what was described as a buccal capsule

using light microscopy. No additional structures enclosing
gut and proboscis were detected. However, the features in
the complex cuticular neck region of the oral disc may
have been what was described as buccal gubernaculum.

Cuticle and molting

The metamorphosis of the tantulus into a new stage takes
place without any normal molting process, which begs the
question how the cuticle can expand in area. The cuticle
sac with parthenogenetic eggs grows directly from the pos-
terior margin of cephalon after the trunk somites are shed
away and can expand up to ten times the size of tantulus
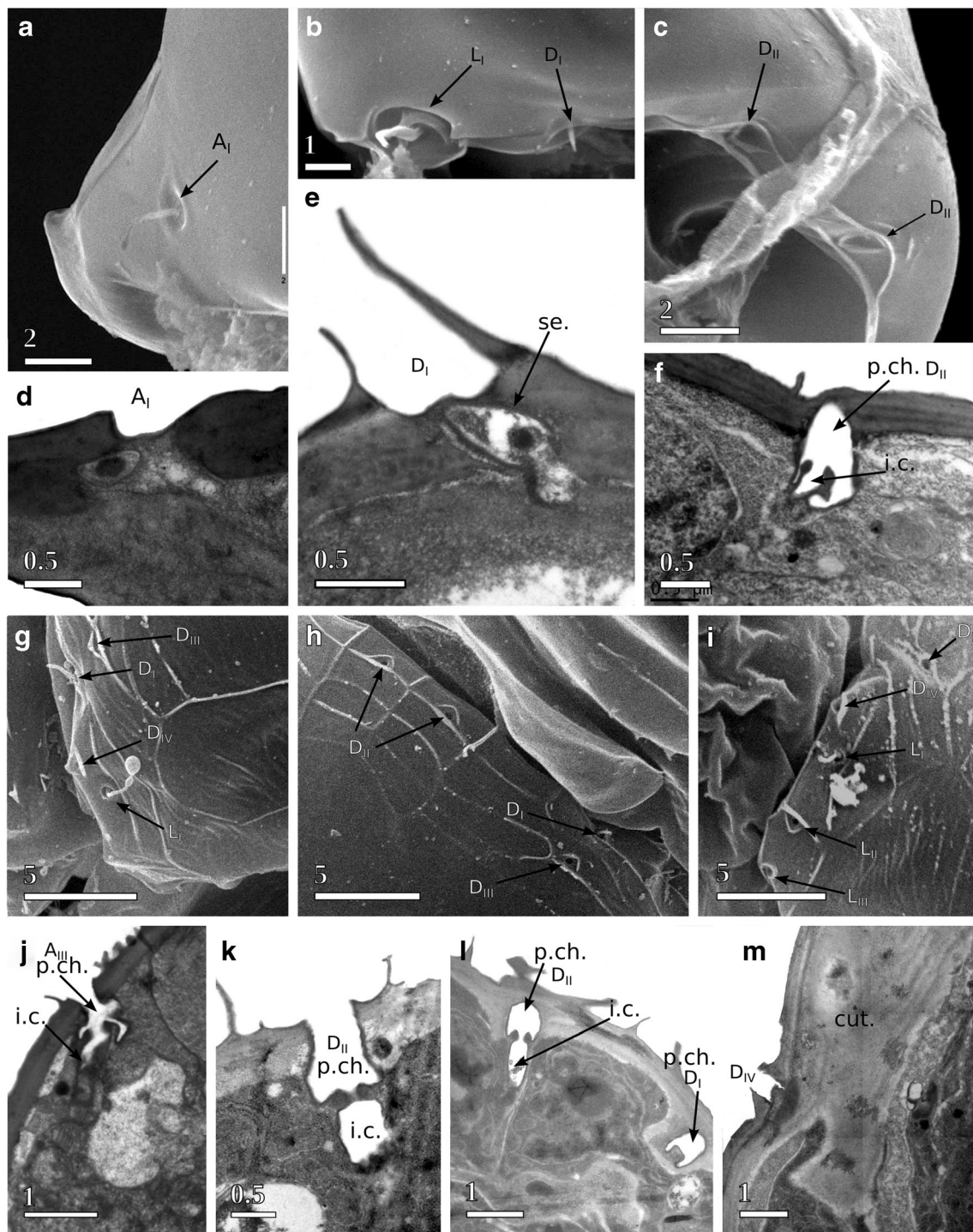larva. We found that the cuticle of the egg sac of the

Fig. 14 Cephalic sensory pores, tantulus larva (**a**–**c**, **g**–**i**—SEM; **d**–**f**, **j**–**m**—TEM). *Microdajus tchesunovi* (**a**–**f**), *Arcticotantulus pertzovi* (**g**–**m**). **a** Anterior part of cephalon, dorsolateral view. **b**, **c** Posterior part of cephalon, dorsal view. **d** Anterior part of cephalon, longitudinal section through A_I pore. **e** Posterior part of cephalon, longitudinal section through D_I pore. **f** Posterior part of cephalon, transverse section through pore chamber and beginning of inner cavity of D_{II} pore. **g**–**i** Posterior margin of cephalon showing simple and sensillate pores. **j** Transverse section through A_{III} pore with outer pore chamber and inner cavity. **k**, **l** Posterior margin of cephalon, transverse section through D_{II} and D_I pores. **m** Posterior margin of cephalon, transverse section through D_{IV} pore. A, D, L, cephalic pores; cut., cuticle; i.c., inner cavity; p.ch., pore chamber; se., sensilla. Scale bars in micrometers

parthenogenetic stage has the same structure as in the cephalon of the tantulus larva so a simple stretching cannot explain the growth of the cuticle. A similar and similarly unexplained expansion of cuticle area without any molting occurs during the growth of rhizocephalan externae (Høeg 1982; Høeg and Lützen 1995).
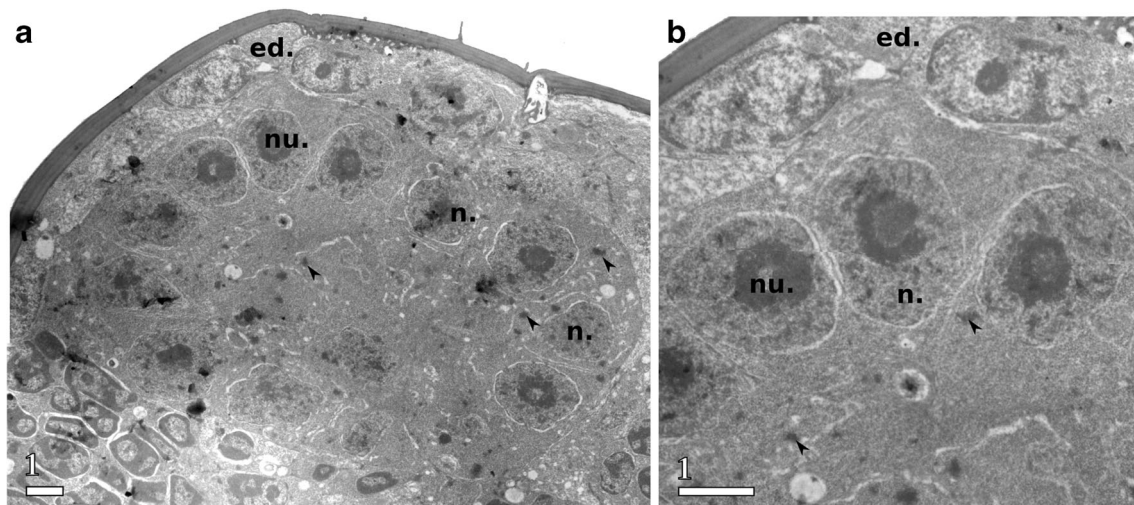
Fig. 15 Ovary (TEM, tantulus larva). *Microdajus tchesunovi*, frontal cross-section through cephalon. **a** Ovary located in posterior part of cephalon (nuage in cytoplasm indicated by arrowheads). **b** Developing oocytes (nuages in cytoplasm indicated by arrowheads). ed., epidermis; n., nucleus; nu., nucleolus. Scale bars in micrometers

Nervous system and sensory structures

The nervous system is greatly influenced by the significant miniaturization of the tantulocarid larva. However, all modifications have their limits. For example, the size of neurite bodies is close to its limit of 1 μm. The number of nervous cells in the cortex layer is very low in comparison with closely related normal size Thecostraca. Neurons' ultrastructure shows typical patterns of miniaturization, such as highly condensed chromatin concentrated along the nuclei walls, reduced size of neuron bodies, tightly packed nervous cells in the cortex layer of the brain, and reduced neuropil, features also observed in other miniature arthropods (Makarova and Polilov 2013).

The tantulus must obviously be able to identify the right host species upon contact and before it commits itself for irreversible attachment, and it seems that the only structures available for this are the putative sensory pores and pits identified here by TEM. The inner structure of such cuticular pores was studied in several crustacean groups by Halcrow (1993), and the pores of the tantulus conforms to a general pattern by having a cuticular chamber and an inner epidermal canal or lumen and by being connected with sensory dendritic processes. Somewhat surprisingly, we did not find any signs of a paraciliary segment that normally is associated with most chemo- and mechanoreceptors in crustaceans (Hallberg and Skog 2011). It is possible that the morphology of the sensory pores seen in the tantulus is the result of the extreme miniaturization of the body. In several species, the free-swimming tantulus larvae have up to four pairs of cuticular filaments on the oral disc (Huys 1991; Huys et al. 1992; Martinez Arbizu and Petrunina 2017), but our TEM study could not prove a sensory function for these structures.

Comparison of Tantulocarida and Thecostraca

The present TEM investigations of the Tantulocarida give us for the first time a chance for comparison of their internal structures with other Crustacea in search of possible homologies. It has for a long time been suggested that Tantulocarida could be closely related to the Thecostraca, and recent molecular phylogenetic studies support this hypothesis and even suggest that they could be nested within this taxon (Khodami et al. 2017; Petrunina et al. 2014). The Thecostraca comprise the assumedly parasitic Facetotecta, the parasitic Ascothoracida, and the Cirripedia that comprise both parasitic and filter-feeding forms (Grygier 1987; Høeg et al. 2009). Although adult thecostracans differ widely in morphology and life style, their monophyly has until now been unchallenged based on very strong evidence from both larval morphology and molecular data (Pérez-Losada et al. 2004; Høeg et al. 2009). Whether or not tantulocarids are nested within Thecostraca, there are numerous spectacular similarities between the two taxa that need to be discussed in order to decide whether or not they represent homologies or convergent evolution: the general life cycle including permanently attached adult stages, the general tagmosis and function of the larval stages, the mode of fixation on a substratum and the sensory structures used in this process, and feeding mechanisms. Finally, the presence of several parasitic groups within Thecostraca obviously raises the question: are Tantulocarids closely related to one of these (Høeg et al. 2009; Pérez-Losada et al. 2009)? Only based on such a comparison together with additional molecular evidence can it be decided if the concept of Thecostraca and its intrinsic phylogeny need to be revised.

General tagmosis and function of the larva

The tantulus larva has the same overall tagmosis and mode of life as found in the cypridoid larva common to all Thecostraca. They all have a short free-swimming life followed by settlement on a substratum leading to the metamorphosis into either parasites or filter-feeding forms. Like the cypris, the tantulus also has six pairs of swimming legs, but more evident homologies cannot easily be found. All thecostracans have a cypridoid larvae sharing a suite of unique characters including (1) six thoracic segments with natatory thoracopods, (2) frontal filaments associated with compound eyes, (3) compound eyes with tripartite crystalline cones, (4) lattice organs in a carapace, and (5) prehensile antennules used in attachment by either mechanical or chemical means (Høeg et al. 2004, 2009). None of these features except the thoracopods are present in the tantulus, but this could of course be due to secondary modification and loss. Although surprisingly stereotyped, the cirripede cyprid can also lose structures through specialization, with perhaps the most spectacular example being the total loss of thoracopods and even the entire thorax in some forms (Kolbasov and Høeg 2007).

Life cycle

Tantulocarids are specialized parasites having a complex life cycle including both sexual and parthenogenetic phases, whereas other Thecostraca only have a sexual phase. The tantulus larva could be evolved as an adaptation for such a complex life cycle and may represent a synapomorphy for tantulocarids. In these terms, we cannot compare tantulus and cypridoid instars. But a cypridoid larva resembles sexual stages in generalized Ascothoracida (Kolbasov et al. 2008; Høeg et al. 2009) in general morphology, including the lattice organs. Therefore, in further studies, we are going to compare external morphology and anatomy of tantulocaridan sexual stages with cypridoid larvae to establish some possible homologies.

Cement adhesion

The Tantulocarida and the Cirripedia are unique in so far as adhesion is assured exclusively by means of chemical substance produced in cephalic glands. In both the tantulus and the cypris of the Cirripedia, this proceeds by a secretion of cement from paired multicellular cement glands that exit first through a cuticular lined secretion chamber and then through long cuticular ducts or canals (Walker 1971; Høeg 1985). But there are also crucial differences. The cypris cement glands exit onto each of the paired antennules whereas the four ducts in the tantulus terminate onto the tip of the eversible and unpaired proboscis. Moreover, the secretion in the cypris is controlled by a muscular sac probably acting as a release valve whereas any such structure is absent in the tantulus. But

despite these differences, a homology cannot be excluded and it would be highly interesting to also compare the chemical properties of the cement in these two taxa in search of possible similarities. There are in fact interesting similarities between the attachment process in the tantulus and at least some of the rhizocephalan cirripedes. In both tantuli and kentrogons of *L. porcellanae*, the larva is at first loosely attached by either proboscis or antennules but is then pulled toward the substratum and firmly glued by renewed cement secretion (Høeg 1985). While this is probably just a convergently evolved trait, it nevertheless underlines the need in both tantulus and cypris/kentrogon to assure a firm attachment for the penetration into the host by means of a stylet.

Lattice organs

To perform their function in locating potential host or surface for the settlement, both cypridoid thecostracan larvae and a tantulus larva need sensory apparatus. In thecostracan cypridoid larvae, the most important sensory organs, aside from eyes, are the sensilla on the antennules and the specialized chemosensory structures in the carapace called lattice organs and considered as a synapomorphic feature for the Thecostraca (Jensen et al. 1994; Høeg et al. 1998; Høeg and Kolbasov 2002). The tantulocarid larva lacks eyes and both antennules and lattice organs, and this leaves the sensory pores and pits as the only possible organs responsible for substratum recognition.

The sensory pores studied here have some resemblance to the lattice organs in having both a cuticular chamber and an inner epidermals part, but in the tantulus, both chamber and pore are devoid of any cellular processes, whereas the chamber of lattice organs contains the paraciliary segment of the sensory cell. Moreover, lattice organs almost always occur in five pairs in the dorsal surface, whereas there is no such fixed number among species of Tantulocarida. Lattice organs are considered one of the key morphological features assuring the monophyly of the Thecostraca and seem not to be purely larval structures as they are also present in adults of some Ascothoracida. It would therefore be of high interest to search for possible homologies also in the sexual stages of the Tantulocarida.

Feeding adaptations

The rootlet system of the Tantulocarida is obviously a unique structure with few parallels in other parasitic crustaceans. Outside the Thecostraca, the only example may be parasitic copepods of the order Siphonostomatoida such as *Rhizorhina* sp. (Kakui 2016). Within Thecostraca, so-called rootlet systems developed three times, but clearly convergently and being different in structure, ontogeny, and function. The best known example is the rootlets of the Cirripedia Rhizocephala, which represents the whole body of the parasite at its endoparasitic stage (interna), and later provides nutrient

to the external reproductive sac. Two genera of parasitic thoracican cirripedes, *Rhizolepas* infesting polychaetes and *Anelasma* infesting sharks, also have rootlets, but they are in both cases much less extensive and develop from the base of the peduncle (Day 1939; Rees et al. 2014).

The stylet Cuticular stylets are developed in several groups of parasitic crustaceans. Fish lice (Branchiura) have a so-called pre-oral stylet, an unpaired cuticular organ with two subterminal openings as part of their mouth apparatus. This stylet is supposedly used for injection of toxins into host tissues, but not for obtaining nutrients (Walker et al. 2011). A much more relevant comparison is with the Cirripedia Rhizocephala. In these, the kentrogon larva has a stylet that is used to clear the way into host tissues for the invasive stage called the vermigon. Having few morphological characters, the stylet in rhizocephalans looks rather similar to that seen in the Tantulocarida, but they differ in both function, development, and the mechanism of penetrating into the host. In rhizocephalans, the stylet is hollow from the base to the tip and used not only to penetrate the host cuticle but also as a passageway for injection of the so-called vermigon larva as an endoparasite into the hemocoel of the host. Moreover, muscles are not involved in the penetration of the stylet of the kentrogon (Høeg 1985; Glenner 2001; Høeg et al. 2012). In tantulocarids, the stylet is operated by muscles and serves for punching a hole in the host cuticle which enables the rootlet system to grow into the host. Moreover, the stylet is present already in the free-swimming tantulus larva, whereas in rhizocephalans, it is only formed after settlement on the host.

Conclusion

The problem of position of Tantulocarida within Crustacea is still wide open. Since the tantulocarids are possibly nested within the monophyletic Thecostraca, there is a need for more molecular analysis and detailed comparison of different life cycle stages. Particular emphasis should be put on the possible homology of the tantulus and the cypris larva and especially on comparison of their cement glands which despite obvious differences might still be homologous. Ultimately, there may be a need to redefine the morphological characters diagnosing the Thecostraca in case the Tantulocarida prove to be a member of this taxon.

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