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The architecture of the joint head cuticle and its transition to the arthrodial membrane in the terrestrial crustacean *Porcellio scaber*

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ABSTRACT

The cuticle of terrestrial isopods is an interesting model for the study of structure-function relationships in biological composite materials. Its organic matrix has a hierarchically organised structure, and type and phase of the mineral compound can vary. The cuticle forms functionally diverse skeletal elements whose properties are adapted to their specific functions. In order to better understand the relation between structure, composition and function of isopod cuticle, we studied the structure and composition of the joint head that is part of the pereiopod's basis. It consists of a central region, whose shape fits well into the joint socket, and an edge region that is connected to the soft arthrodial membrane and protects the central region from mechanical load. The cuticle architecture of the joint head has local variations in structure and composition. In the central region the cuticle is similar to the previously published tergite cuticle. High concentrations of amorphous calcium phosphate are located in the endocuticle suggesting a coexistence with amorphous calcium carbonate. The edge region has an unexpected organisation characterised by thickening of the epi- and exocuticle and an unusual unidirectional orientation of chitin-protein fibrils within the endocuticle. The concentrations of phosphate are considerably higher than in the central region. The overall differentiation in the cuticular architecture of the edge in comparison to the central region reflects the adaptation to mechanical strains the cuticle has to sustain during contraction of extensor muscles, and to the structural and compositional transition from the edge to the connecting arthrodial membrane.

1. Introduction

The integument of arthropods secretes a cuticle that surrounds the whole animal. The cuticle is an exoskeleton, which combines the function of an outer barrier to environmental strains with that of a support for the overall body and the forces that occur during muscle contraction. In general, the arthropod cuticle structure comprises about seven hierarchical levels (Fabritius et al., 2009; Nikolov et al., 2011). It consists from distal to proximal of the four principal layers; the epi-, exo-, endocuticle and membranous layer. The epicuticle is the outermost layer of the cuticle forming surface structures such as scales and epicuticular hairs. It is thin and contains lipids, proteins and waxes and can serve as a barrier against water loss. In the exo-, endocuticle and membranous layer the general architecture is of a twisted plywood structure (Bouligand, 1972). This structure arises from the arrangement of fibres running parallel to one another in planes, and from one plane to the next the fibres pile up at an angle forming stacks. The stacking height is defined by the distance between two planes separated by a twist of about 180°. The fibres are formed by fibrils, which are chains of α-chitin crystallites coated with protein in a helical manner (Blackwell and Weih, 1980). The chitin crystallites consist of 19 antiparallel-oriented chitin molecules (Carlström, 1957). In Crustacea the exo- and endocuticle is reinforced with a mineral phase of Mg-calcite, amorphous calcium carbonate, and minor amount of amorphous calcium phosphate (Boßelmann et al., 2007; Dillaman et al., 2005; Hild et al., 2008; Neues et al., 2007, 2011; Seidl et al., 2011). This makes the crustacean cuticle a biological composite material with outstanding mechanical properties.

Depending on the organisation of limbs and body segments the cuticle of an arthropod can be subdivided into various skeletal elements. Although the general scheme of cuticle architecture is similar, structure as well as composition of the cuticle is not uniform but adapted to the various environmental and internal loads the cuticle has to sustain in different regions of the body. This is of high interest for the development of biomimetic composite materials in which structural characteristics modulate their mechanical properties.

The type and magnitude of strains the cuticle has to sustain depend on the specific function of a skeletal element as well as the

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behaviour and habitat of the animal. The relation between behaviour and habitat on the one hand, and structure and composition on the other has been recently investigated in a series of comparative studies on the tergite cuticle of isopods (Becker et al., 2005; Hild et al., 2008, 2009; Neues et al., 2007; Seidl et al., 2011). However, comparative studies on the cuticle of different skeletal elements within the same isopod species are rare.

In the present study we analyse the structure and composition of the joint head cuticle with special emphasis on the transition towards the arthrodial membrane. The joint head is part of the basis that is the most proximal of seven articles in the isopod pereiopod. Together with the flexible arthrodial membrane it forms the articulation between the pereiopod basis and the coxal plate on the lateral side of the animal body. We use transmission electron microscopy (TEM), field emission scanning electron microscopy (FE-SEM) and low voltage scanning transmission electron microscopy (STEM) for the structural analysis, as well as X-ray microprobe analysis (EPMA) and scanning confocal μ-Raman spectroscopic imaging (SCμ-RSI) for the chemical analysis. The results show that the joint head has two distinct regions; a central region in which the cuticle is similar to that in the tergites of *Porcellio scaber* (Hild et al., 2008; Seidl and Ziegler, 2012) and a peculiar edge region that deviates from the cuticle in the central region in both structure and chemical composition.

2. Materials and methods

2.1. Animals

P. Scaber Latreille, 1804 were collected from local biotopes and kept in plastic containers filled with moist soil and tree barks. They were fed with potatoes, carrots and dry oak leaves. Only animals without any signs of moult (Neues et al., 2011) were used for the study.

2.2. Transmission electron microscopy (TEM)

Joints were dissected in a solution containing 0.5 mol L^{-1} EDTA, 2.5% glutaraldehyde, 2% paraformaldehyde and 0.25 mol L^{-1} HEPES at pH 7.8 and incubated in the same solution for 4 days at 4 °C for simultaneous fixation and decalcification. Then the joints were washed three times in bi-distilled water for 10 minutes each, and postfixed for 1 hour in an aqueous solution of 1% OsO_4 and 0.8% $K_4[Fe(CN)_6]$. Samples were then washed 3 times with bi-distilled water for 10 minutes each, dehydrated in an ascending series of isopropanol, block-contrasted by 0.5 wt% uranyl acetate in ethanol, washed 3 times in isopropanol and twice in acetone for approximately 4 minutes each. Joints were embedded in EPON resin and 50–70 nm thick sections were cut with an Ultracut ultramicrotome (Leica, Germany). The sections were placed on carbon-coated formvar films on copper hole grids, stained for 10 minutes with 2% uranyl acetate and for 1 minute with lead citrate, and viewed with a Jeol-1400 TEM at 120 kV.

2.3. Scanning electron microscopy (SEM)

Joint heads were dissected in 100% methanol to prevent dissolution of ACC (Becker et al., 2003). To remove residual saline samples were washed in bi-distilled water for 2 s, transferred quickly to methanol to remove water, and then left to air-dry. The dry joint heads were glued onto aluminium holders and then either cleaved or polished. Cleaved inner faces of the cuticle were achieved by breaking open the joints with a razor blade either by hand or with the help of a Leica micromanipulator. To polish the joint heads, we used an Ultracut ultramicrotome (Leica). Glass knives were used first to cut thicker sections of 200–300 nm until a desired plane within the joint head was exposed, then a diamond knife (Diatome, Switzerland) was advanced successively by 70, 50, 30, 20, 15, and 10 nm for at least 15 cuts each (Fabritius et al., 2005). To reveal in detail the organisation of organic fibres some of the polished samples were etched at pH 8 in a solution containing 0.1 mol L^{-1} HEPES and 2.5% glutaraldehyde. After 20 s of etching the samples were washed 3 times in 100% isopropanol, 10 min each, and then critical point dried (Seidl et al., 2011). All of the cleaved, polished, and etched samples were rotary shadowed at an angle of 45° with 3–4 nm platinum using a BAF 300 (Balzers, Liechtenstein). Samples were analysed using a Hitachi S-5200 FE-SEM at an acceleration voltage of 4 kV and an emission current of 10 μA.

2.4. Low voltage scanning transmission electron microscopy (STEM)

Polished specimens prepared as described above were cut dry using an ultra 35° diamond knife (Diatome, Switzerland) (Seidl et al., 2011) to get 40–70 nm thick sections of non-demineralised cuticle. Sections were transferred to carbon-coated formvar films on 300 mesh copper EM-grids and coated with 3 nm of carbon. For dark field STEM we used the Hitachi S-5200 FE-SEM equipped with a STEM detector at an acceleration voltage of 30 kV and an emission current of 20 μA.

2.5. Electron probe microanalysis (EPMA)

The electron probe microanalysis was performed on polished planes coated with 3 nm of carbon. The FE-SEM (Hitachi S-5200) equipped with a Phoenix (EDAX) X-ray detector system and GENESIS software were used for the analysis. The FE-SEM was set to an acceleration voltage of 20 kV. Elemental maps were recorded at a pixel resolution of 512 × 400 and a dwell time of 200 μs per pixel leading to 88 full scans per hour. For every pixel a full spectrum was recorded and stored (spectral mapping). For each scan spectra were integrated for every pixel allowing for standard less background-corrected calculation of atomic ratios for selected regions within the maps. A drift correction implemented in the GENESIS software was used to avoid reduction of spatial resolution by specimen drift.

2.6. Scanning confocal μ-Raman spectroscopic imaging (SCμ-RSI)

SCμ-RSI was performed on polished joint heads. A scanning confocal Raman microscope from WiTec (Alpha-300R) was used, operating with an NdYag laser at 532 nm at an intensity of 8 mW. A Nikon (50×, air, NA = 0.8) objective lens and a piezo- driven scan stage ensure a lateral spatial resolution of 300 nm (*x* and *y* axis). The spatial resolution in *z*-axis was about 500 nm. The Rayleigh peak was suppressed by using a holographic edge filter. Applying a diffraction grating with 600 grooves per cm a spectral resolution of approximately 3 cm^{-1} was achieved for a full spectrum ranging from 0 to 3750 cm^{-1}. The incident light was polarized either by 0° ($P_{0°}$) or 90° ($P_{90°}$).

All spectra were recorded with the WiTec-Control acquisition software (WiTec GmbH, Ulm, Germany). Samples were imaged with a raster of 9 pixels per 1 μm^2. At each pixel a full Raman spectrum was recorded with an integration time of 0.5 s. Employing the WiTec-Project software (Version 2_08, WiTec GmbH, Ulm, Germany) (Schmidt et al., 2005) we integrated for each pixel the band for the external (lattice) vibration between 260–310 cm^{-1} (*ΣCalcite*) and that of the band for symmetric stretching vibration of carbonate between 1070–1100 cm^{-1} (*ΣCarbonate*). The C-H and CH_2 stretching vibration between 2800–3100 cm^{-1} (*ΣOrganic*) [chitin and protein] and the phosphate vibration between 930 and 980 cm^{-1} (*ΣPhosphate*) were also integrated. For imaging of the