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Ultrastructure and mineral composition of the cornea cuticle in the compound eyes of a supralittoral and a marine isopod

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ABSTRACT

The cuticle of the cornea in Crustacea is an interesting example of a composite material compromising between two distinct functions. As part of the dioptric apparatus of the ommatidia within the complex eye it forms transparent micro-lenses that should as well maintain the mechanical stability of the head capsule. We analyzed the ultrastructure and composition of the isopod cornea cuticle of the terrestrial species *Ligia oceanica* and the marine *Sphaeroma serratum*. We used a variety of tissue preparation methods, electron microscopic techniques as well as electron microprobe analysis and Raman spectroscopic imaging. The results reveal various structural adaptations that likely increase light transmission. These are an increase in the thickness of the epicuticle, a reduction of the thickness of the outer layer of calcite, a spatial restriction of pore canals to interommatidial regions, and, for *S. serratum* only, an increase in calcite crystal size. In both species protein–chitin fibrils within the proximal exocuticle form a peculiar reticular structure that does not occur within the cuticle of the head capsule. In *L. oceanica* differential mineralization results in a spherically shaped interface between mineralized and unmineralized endocuticle, likely an adaptation to increase the refractive power of the cornea maintaining the mechanical stability of the cuticle between the ommatidia. The results show that the habitat and differences in the general structure of the animal's cuticle affect the way in which the cornea is adapted to its optical function.

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1. Introduction

Mineralized biological composite materials like the crustacean cuticle have attracted increasing interest because of their outstanding mechanical properties that are well adapted to their biological function and their high potential in biomimetic research and technical applications. Like all arthropods, Crustacea have a protective exoskeleton, the cuticle, which also provides support and sites for muscle attachment. It consists of an organic matrix composed of chitin–protein fibers and an inorganic phase consisting of calcite, amorphous calcium carbonate and minor amounts of amorphous calcium phosphate (Becker et al., 2005; Boßelmann et al., 2007; Greenaway, 1985). The organic phase is hierarchically organized (Fabritius et al., 2009; Nikolov et al., 2011). It consists of α-chitin crystals 2.5–3 nm in diameter and about 300 nm length (Carlström, 1957; Vincent and Wegst, 2004) that are coated with proteins in a helical manner (Blackwell and Weih, 1980). These chitin protein fibrils form planes that are stacked upon each other at an angle forming a twisted plywood structure (Bouligand, 1972). The distance between planes in which the fibril orientation is twisted by about 180° is called stacking height. The stacks form the mineralized outer exo- and inner endocuticle and often an unmineralized thin membranous layer. The outermost epicuticle consists of hydrocarbons and waxes, and is devoid of chitin. The cuticle constitutes skeletal elements whose mechanical properties are adapted to their specific function and the habitat of the animal (Fabritius et al., 2009, 2012; Romano et al., 2007; Sachs et al., 2006). The cuticle contains vertically running pore canals (Compère and Goffinet, 1987a). They result from extensions of the hypodermis that forms the cuticle. The extensions secrete fibrils that follow the direction of the pore canals. After retraction of the cellular extension they leave the pores that may later be filled with mineral or remain open (Compère and Goffinet, 1987a,b). Pore canals are thought to provide the path by which substances are transported for the repair of surface cuticle and for calcification (Giraud-Guille, 1984b; Roer and Dillaman, 1984;

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Roer, 1980; Travis, 1965), and affect the mechanical properties of cuticle (Fabritius et al., 2009).

The cuticle that forms the cornea in the arthropod compound eye requires adaptations to its function in vision, in addition to mechanical stability. Therefore, the cornea is an interesting example of cuticle with a bimodal function. Arthropod eyes consist of repeating functional-units called ommatidia. Their number within the eye varies considerably from species to species. They bear light sensitive retinula cells and a dioptric apparatus that consists of a crystal cone and the cuticular cornea. The crystal cone is an organic structure below the cornea that transmits light on the light receptive region (rhabdome) of the sensory cells. The cornea acts as a biconvex lens and allows transmission of light rays to the light sensitive sensory cells of the retina. Past studies on crustacean eyes have focused mainly on the internal structures of the eye but studies on the corneal cuticle are very limited (Edwards, 1969; Keskinen et al., 2002; Nemanic, 1975; Nilsson, 1978, 1983). Thus, in order to understand more on how the cuticular architecture of the cornea is adapted to their functions and the habitat of the animal, we investigated the ultrastructure and chemical composition of the cornea in the compound eyes of the terrestrial isopod species *Ligia oceanica* and the marine isopod *Sphaeroma serratum*. The two species differ in the thickness and strength of their general cuticle and the number of ommatidia, which is about 7 times larger in *L. oceanica* than in *S. serratum*, suggesting that vision plays a larger role in the former.

We used transmission electron microscopy (TEM), field emission scanning electron microscopy (FE-SEM), low voltage scanning transmission electron microscopy (STEM) and optical microscopy for the structural analysis, as well as electron probe microanalysis (EPMA) and scanning confocal μ-Raman spectroscopic imaging (SCμ-RSI) for the chemical analysis. To identify characteristic features of the cornea cuticle, the structures were compared with those of the head capsule when necessary. The results show that the cornea cuticle of both species has structural adaptations compromising between optical and mechanical properties.

2. Materials and methods

2.1. Animals

L. oceanica (Linnaeus, 1767), were obtained from the Biological Institute Helgoland and kept in a plastic container, containing little seawater, pieces of wood and rocks. The animals were fed with 'Tetramin' standard fish feed. The marine species *S. serratum* (Fabricius, 1787) was originally received from Prof. Jasna Štrus (University of Ljubljana, Slovenia) and then bred in a seawater aquarium. They were fed with 'Multifit' standard fish food. Both species were kept at ambient light and temperature conditions. Cornea cuticle, together with adjacent cuticle of the head capsule were dissected in 100% methanol to avoid possible crystallization of the amorphous $CaCO_3$ phase (Becker et al., 2003). Cuticle samples were washed for one second in bi-distilled water to remove body fluids and then for a few seconds in 100% methanol to remove the water. After air drying eye cuticles were either used immediately or stored at −20° C until further use.

2.2. Polarized light microscopy

Freshly prepared soft-tissue free transparent eye cuticles were viewed between crossed polarization filters of a light microscope (Axiophot I, Fa. Zeiss) to visualize the distribution and orientation differences of calcite in the eye cornea and the surrounding head capsule cuticle.

2.3. Transmission electron microscopy (TEM)

Freshly prepared cuticle samples were decalcified and their organic matrices simultaneously fixed by incubation 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 for approximately 5 days for *L. oceanica* and 10 days for *S. serratum* at 4 °C. Samples were postfixed, embedded in EPON and ultrathin sections were obtained as described previously (Ruangchai et al., 2013). Sections were examined at 120 kV using a Jeol-1400 TEM equipped with an Olympus digital camera (2000 × 2000 pixels) and iTEM software.

2.4. Scanning electron microscopy (SEM)

Eye samples were either cleaved or polished. To obtain polished planes through the cuticle, samples were first glued on aluminium holders and polished using a diamond knife (Diatome) as described previously (Fabritius et al., 2005). Some of the polished samples were etched either at pH 8.0 or at pH 6.5 in a solution containing 0.1 mol L^{-1} HEPES and 2.5% glutaraldehyde. After 10–20 s of etching, the samples were washed 3 times in 100% isopropanol, for 10 min to remove the aqueous solution (Seidl et al., 2011) and then critical point dried (CPD 030, Bal-Tec, Liechtenstein). Cleaved faces through the cuticle were achieved by breaking manually with forceps or with the help of razor blades mounted on a Leica micromanipulator. All the cleaved, polished and etched samples were rotary shadowed with 3.5–4 nm platinum at an angle of 45° using a freeze fracture device (BAF 300, Balzers, Liechtenstein). Samples were analyzed using a FE-SEM (Hitachi S-5200) at an acceleration voltage of 4 kV and an emission current of 10 μA. Whole heads were sputter-coated with 20 nm gold/palladium (Balzers MED 010) and analyzed in a DSM 962 SEM (Zeiss, Germany) at an acceleration voltage of 20 kV.

2.5. Low voltage scanning transmission electron microscopy (STEM)

Sagittal ultrathin sections of intact (non-demineralized) eye cuticles were cut dry from polished samples using an ultra 45° diamond knife. The sections were sandwiched between carbon coated formvar films on 300 mesh grids and flattened by the weight (0.1 N) of a brass rod. Sandwiched grids were separated and sections were coated with an approximate 6 nm thick layer of carbon (BAF 300, Balzers, Liechtenstein). Dark field STEM was performed using a FE-SEM (Hitachi S-5200) equipped with an STEM detector at an acceleration voltage of 30 kV and an emission current of 20 μA.

2.6. Electron probe microanalysis (EPMA)

Electron probe microanalysis of polished surfaces coated with a 3 nm thick layer of carbon was performed with a FE-SEM (Hitachi S-5200) equipped with a Phoenix X-ray detector system and GENESIS software (EDAX). The microscope was set to 20 kV acceleration voltage and 10 μA emission current. 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). At each scan, spectra were integrated for every pixel allowing for standard-less background-corrected calculation of atomic ratios for selected regions within the maps and to generate background corrected maps. A drift correction implemented in the GENESIS software was used to avoid reduction of spatial resolution by specimen drift.

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