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### PAPER

# Functional adaptation of crustacean exoskeletal elements through structural and compositional diversity: a combined experimental and theoretical study

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### Abstract

The crustacean cuticle is a composite material that covers the whole animal and forms the continuous exoskeleton. Nano-fibers composed of chitin and protein molecules form most of the organic matrix of the cuticle that, at the macroscale, is organized in up to eight hierarchical levels. At least two of them, the exo- and endocuticle, contain a mineral phase of mainly Mg-calcite, amorphous calcium carbonate and phosphate. The high number of hierarchical levels and the compositional diversity provide a high degree of freedom for varying the physical, in particular mechanical, properties of the material. This makes the cuticle a versatile material ideally suited to form a variety of skeletal elements that are adapted to different functions and the eco-physiological strains of individual species. This review presents our recent analytical, experimental and theoretical studies on the cuticle, summarising at which hierarchical levels structure and composition are modified to achieve the required physical properties. We describe our multi-scale hierarchical modeling approach based on the results from these studies, aiming at systematically predicting the structure-composition-property relations of cuticle composites from the molecular level to the macro-scale. This modeling approach provides a tool to facilitate the development of optimized biomimetic materials within a knowledge-based design approach.

### 1. Introduction

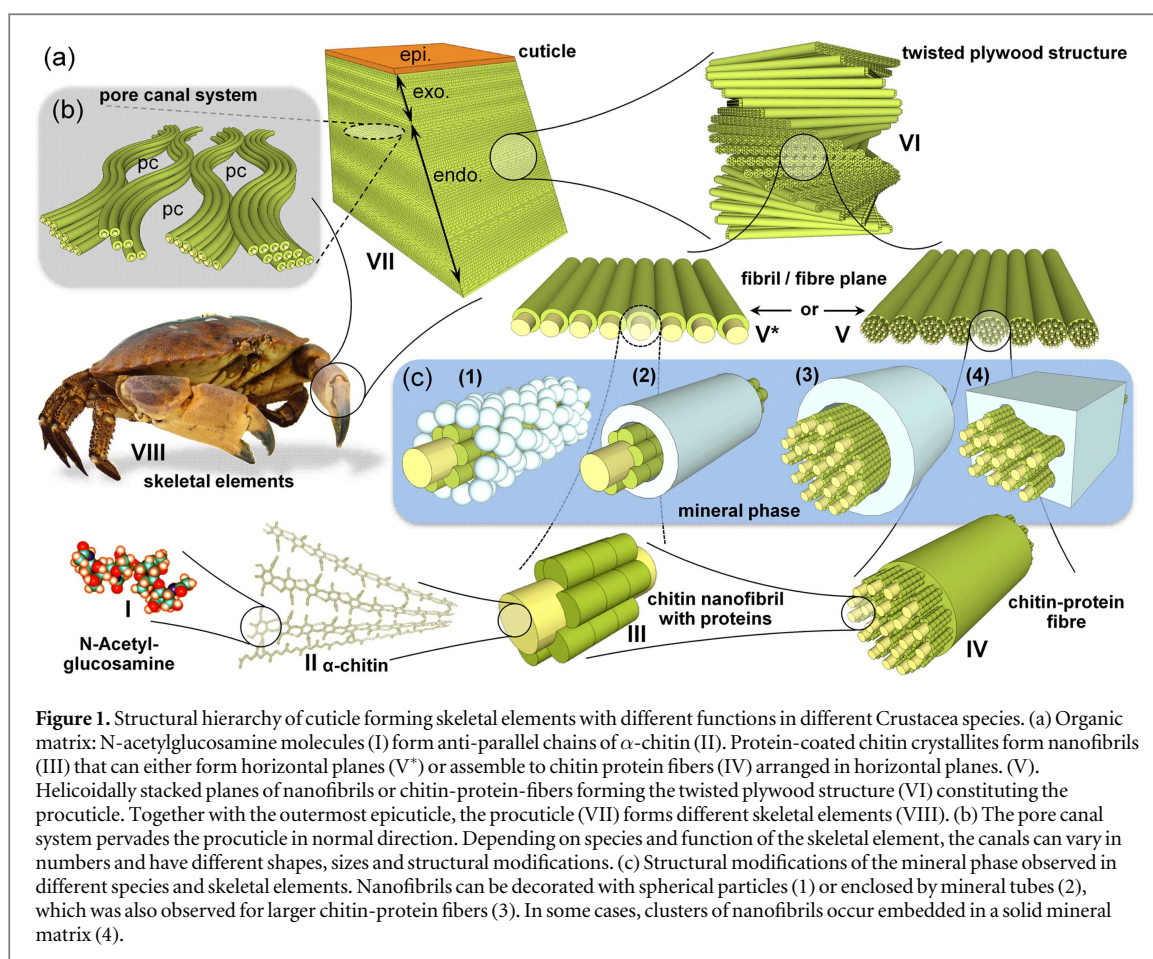
One of the most characteristic features of the Arthropoda in general is that their integument is formed by a cuticle that has evolved into an exoskeleton. The evolutionary success of this group, which is the most species-rich within the animal kingdom, has led to an enormous variety of body plans that are specifically adapted to different ways of life in different habitats

(Brusca 2000). From a phylogenetic point of view, the ancestral arthropods were segmented animals. Over millions of years of evolution, the originally uniform body segments have more and more specialized to fulfil different functions, leading to a large diversification in morphology (Edgecombe 1998). The cuticle is secreted by the hypodermis cells, which are ontogenetically of ectodermal origin and cover not only the exterior surface of the animals, but also internal organs

such as parts of the digestive system or the respiratory system of insects, the tracheae. In consequence, the cuticle is equally present all over the arthropod body. Owing to its function as an exoskeleton, the cuticle forms skeletal elements whose morphologies follow the genetically predetermined segmentation of the body. They have a large variety of different functions related to the body plan, habitat, and the behavior of the animal (Hadley 1986, Fabritius *et al* 2009). Fulfilling these functions requires correspondingly adapted physical properties of the cuticle forming the skeletal element. Depending on the skeletal element, these physical properties are very diverse. The most prominent function of the cuticle is to provide body parts a stable shape and mechanical resistance. Cuticle parts forming the outer protective shell of crustaceans require optimized mechanical properties not only to ensure protection against predation, but also to withstand internal forces exerted by attached muscles that enable movement of skeletal elements. Appendages specialized for catching and killing prey like claws or chelae require tailored properties such as extreme hardness or fracture toughness, that are adapted to the kind of prey a species feeds on (Weaver *et al* 2012). This also applies to skeletal elements like the mandibles and other mouthparts that function in holding, cutting and grinding of food items (Bentov *et al* 2012, Huber *et al* 2014, 2015). Between skeletal elements, the cuticle forms a variety of different joint structures to enable controlled relative movements between them. In addition to a high structural stiffness, properties like friction reduction and wear resistance play an important role for joints (Ruangchai *et al* 2013). The remaining space between skeletal elements necessary for movements is also closed by the cuticle. These elastic arthroal membranes seamlessly interconnect the mostly rigid skeletal elements (Ruangchai *et al* 2013). The cuticle also forms the cornea lenses as parts of the compound eyes of arthropoda, where it has to be both transparent and mechanically resistant (Alagboso *et al* 2014). From an engineering standpoint, it is very interesting how one material like the cuticle can be tailored to have such a wide variety of properties that enable it to perform such different functions. Additionally, as demonstrated by the last example, the cuticle often has to perform more than one function. The most obvious one is that, independent of exoskeletal functions, the cuticle has to seal the body against the environment, preventing e.g osmotic effects or fouling in aquatic species or desiccation in terrestrial species (Hadley 1986). It is a very interesting question how a material can be tailored to achieve the best compromise between different functions. Since the cuticle is a continuous tissue, it also provides transitions between e.g. mineralized skeletal elements and arthroal membranes connecting them. Such transitions are of particular interest since the physical properties change dramatically from one structure to the other. Achieving such variability in the physical

properties of a single material requires a high degree of freedom in the structural and compositional organization. The keys to the cuticle being capable of performing such different tasks are certainly its hierarchical organization based on the structural polysaccharide molecule chitin and its composite nature (figure 1) (Raabe *et al* 2006, Fabritius *et al* 2012). This structural hierarchy offers many possibilities for varying the structural organization.

While the general architecture of the crustacean cuticle is well established (Bouligand 1970, Giraud-Guille 1984, Hadley 1986, Weiner and Addadi 1997, Giraud-Guille 1998), many recent studies have shown that the classical hierarchy of the organic matrix is frequently varied in functionally adapted skeletal elements both in number of levels as well as in terms of structural arrangement and composition in individual levels. This was observed both within one species as well as in homologous parts of different species (Hild *et al* 2008, Fabritius *et al* 2009, Hild *et al* 2009, Seidl *et al* 2011, Fabritius *et al* 2012, Seidl *et al* 2012, Ruangchai *et al* 2013, Alagboso *et al* 2014, Huber *et al* 2014, Huber *et al* 2015). The lowest level for the organic constituents is the sugar N-acetylglucosamine (figures 1(a)(I)), whose linear  $\beta$ -1,4-linked polymer chains arrange in an anti-parallel fashion forming orthorhombic-symmetry  $\alpha$ -chitin crystallites (figures 1(a)(II)). The highly ordered crystalline structure of  $\alpha$ -chitin originates from hydrogen bonds of four hydroxyl groups and two amide groups in each repeating unit (Neville *et al* 1976).  $\alpha$ -chitin is the predominant crystalline chitin polymorph occurring in crustacean cuticle. Typically, the  $\alpha$ -chitin crystallites form nanofibrils with a polygonal section contour that consist of 18–25 chitin chains with a diameter of about 2.5 nm and lengths of about 300 nm (Neville *et al* 1976). The nanofibrils are always confined by an approximately 1.5 nm thick sheath of proteins (figures 1(a)(III)). Individual chitin nanofibrils are the smallest units that are always present in cuticle independent of species, skeletal element or function. They can assemble to chitin-protein fibers (figures 1(a)(IV)) with diameters between 50 and 250 nm. The chitin-protein fibers are arranged parallel to each other forming planes (figures 1(a)(V)). However, chitin-protein fibers are not formed in every species. If that is the case, the higher levels are directly assembled from nanofibrils (figures 1(a)(V\*)). Irrespective of whether the planes are formed by individual nanofibrils or larger clusters and/or fibers they usually form stacks where the fiber orientation changes helically, thereby creating a twisted plywood structure (figures 1(a)(VI)). These stacks form the three main layers of the cuticle: exocuticle, endocuticle and membraneous layer. They can differ in the density of fiber components, the number of planes forming a microscopical stack and the rotation angle between superimposed fiber planes forming the twisted plywood. Together with the external thin and waxy epicuticle, which consists mainly of long chain



hydrocarbons, esters of fatty acids, and alcohols they constitute the bulk cuticle (figures 1(a)(VII)) that forms the skeletal elements (figures 1(a)(VIII)).

In addition to composition and organization of the organic matrix, the physical properties of the cuticle are also influenced by the pore canal system. Pore canals are formed by cellular extensions during secretion of the organic phase of the cuticle. They retract during the successive deposition of chitin layers leaving canals with an almond-shaped cross section that pervade the cuticle perpendicular to its surface (figure 1(b)). They are present in all skeletal elements and serve as transport pathways for the epithelial cells. In different cuticles and even in different layers of the same cuticle the canals vary in number, dimensions and shape. Depending on its organization, in Crustacea the pore canal system serves not only for transport but can also fulfil mechanical tasks (Sachs *et al* 2008). In some cases, pore canals are reinforced by internal mineralized fibrils (Fabritius *et al* 2012) or even completely mineralized (Seidl *et al* 2012).

In contrast to most other major taxa within the Arthropoda, the Crustacea can incorporate biominerals into their cuticle. Thus, they dispose of additional options to tailor its properties by forming organic-inorganic composite materials. Crustacea are known to use a variety of mineral phases. The main mineral is calcium carbonate that mostly occurs in the

form of Mg-calcite and amorphous calcium carbonate (ACC). However, also calcium phosphates like amorphous calcium phosphate (ACP), hydroxyapatite and even fluoroapatite have been identified in crustacean cuticle (Lowenstam and Weiner 1992, Becker *et al* 2005, Boßelmann *et al* 2007, Neues *et al* 2007, Al-Sawalmih *et al* 2008, Bentov *et al* 2012, Weaver *et al* 2012, Amini *et al* 2014). In addition to the type of mineral, Crustacea can precisely control the localization and amount of deposited mineral, from the level of entire skeletal elements down to specific layers of the cuticle, which can contain several different minerals that are co-localized. In most but not all cases the mineral is aligned along the organic fibers and thus follows the hierarchical organization of the organic matrix (Fabritius *et al* 2009, 2012). In addition, the animals also control the morphology of the mineral phase. Structurally, four major different mineral morphologies have been described: On the nanofibril level, mineral occurs either in the form of small, 20–50 nm thick spherical particles located around the nanofibrils (figures 1(c)(1)) (Fabritius *et al* 2009) or in the form of solid tubes enclosing the nanofibrils (figures 1(c)(2)) (Seidl *et al* 2011, Ruangchai *et al* 2013). On the chitin-protein fiber level, mineral can occur as tubes enclosing the fibers (figures 1(c)(3)) (Fabritius *et al* 2011, 2012) or as large mineral blocks surrounding irregularly shaped clusters of nanofibrils

(figures 1(c)(4)) (Seidl *et al* 2011, Ruangchai *et al* 2013). Lastly, it has been shown that Crustacea are able to control not only the crystalline phase of the bulk minerals, but also the local crystallographic properties, e.g. the orientation and texture of calcite (Al-Sawalmih *et al* 2008, Seidl *et al* 2012, Huber *et al* 2015).

The variety of possible compositions, the variability in the hierarchical organization, including quantitative and geometrical aspects as well as the resulting variety of physical properties and their combinations makes the crustacean cuticle an interesting model material for the development of novel bio-inspired materials with tailored properties that can perform multiple functions. To explore this potential, it is necessary to understand the design principles of such materials with specific functions. This requires detailed knowledge about the relationships between their structure, composition and the resulting physical properties. For this, we applied a large variety of experimental methods and techniques to study microstructure, chemical composition and mechanical properties and behavior of cuticle from different skeletal elements of two prominent groups of crustaceans, the Isopoda where we focused on a variety of terrestrial species from the taxon Oniscidea and one aquatic species, and the Decapoda where we concentrated on large marine species like the lobster *Homarus americanus* and the crab *Cancer pagurus*. In practice, however, it became clear that it is not possible to study the structure-property relationships for each hierarchical level due to methodological constraints and size limitations. To overcome this, we developed a numerical multiscale model based on the experimental results that can describe and investigate material properties systematically from the atomic to the macroscopic scale (Friak *et al* 2013). This approach cannot only be used to model the mechanical properties of different bulk biological materials (Fratzl and Weinkamer 2007, Nikolov and Raabe 2008, Nikolov *et al* 2010), but also those of individual constituents on increasingly complex structural hierarchy levels (Buehler 2008, Tang *et al* 2009). Modeling the structure and properties on each level of structural hierarchy provides the flexibility to account for the inherent local structural and compositional variations of the cuticle, allowing to study the tolerance of e.g. the mechanical properties to changes with a high resolution. Once validated, such a model can easily be modified to structure and composition of skeletal elements with different functions or analogous skeletal elements of the same species by changing parameters at the appropriate scale levels. Careful comparison of model predictions and experimental results allow even to obtain reliable estimates for the properties of constituents that are unknown, poorly characterized or experimentally not accessible. Using our model, we were able to identify the most relevant key ingredients of cuticle with respect to mechanical properties and the tolerance of the mechanical behavior of cuticle against structural

variations, which makes it a very useful tool for biomimetic approaches (Nikolov *et al* 2015).

In this review, we want to summarize our recent experimental and theoretical work on crustacean cuticle from a variety of species and skeletal elements with different functions. Our focus lies on comparing their structural and compositional characteristics, discussing the different function-related adaptive strategies we observed, and providing examples and guidelines how the obtained data can be used to study the structure-property relations in the cuticle by multi-scale modeling.

## 2. Structural and compositional diversity of the crustacean cuticle

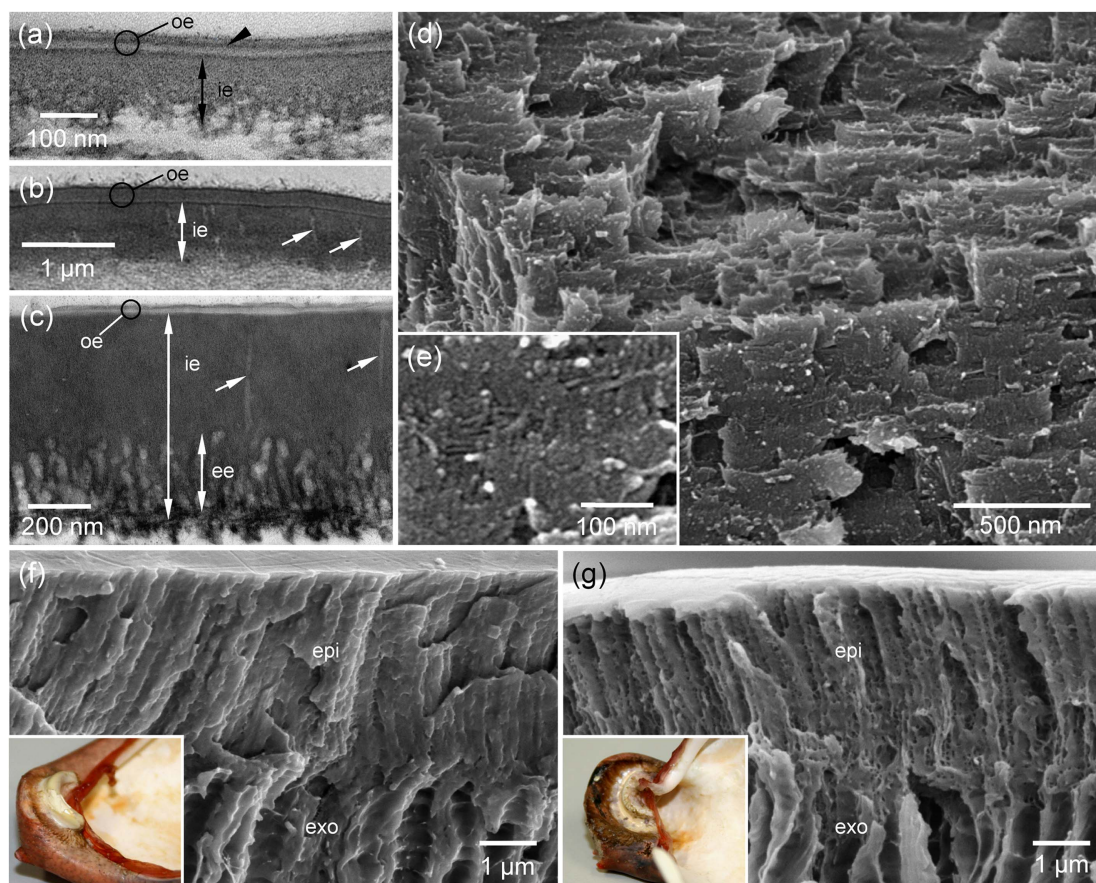
### 2.1. The main sublayers of the cuticle

#### 2.1.1. Epicuticle

In isopods the epicuticle consists of an outer and an inner layer. It is the outermost layer of all skeletal elements and forms surface structures like scales and sensillae. In tergites the thickness of the outer layer ranges between 9 and 50 nm, with the thinnest in small species of the family Trichoniscidae (Vittori *et al* 2012, Vittori and Štrus 2014). The thicker outer epicuticles occur in larger species like *Ligia oceanica*, *Tylos europaeus*, and *Porcellio scaber* (Seidl *et al* 2011, Seidl *et al* 2012, Ruangchai *et al* 2013, Alagboso *et al* 2014) (table 1). In terrestrial species the outer epicuticle appears striated due to one or multiple osmiophilic waxy layers (figure 2(a)). These are thought to be the main barrier against evaporative water loss of the animals (Hadley and Warburg 1986, Compère 1990). This view, however, was recently challenged by Vittori and Štrus (2014), comparing troglodytic species that should be less vulnerable to desiccation than epigeal isopod species. Unexpectedly, there was no correlation between the number of waxy layers and habitat. Therefore, Vittori and Štrus (2014) suggested that the waxy layers may serve other functions that may be ‘...related to the possible presence of epibionts, structural support of ornamentations such as epicuticular scales, or wetting of the cuticular surface’. The inner layer of the epicuticle is of homogeneous appearance. In most small species of the Trichoniscidae, its thickness is similar to the outer layer. However, in larger isopod species it is 2–7 times as thick in the general load-bearing cuticle like the tergites or the head capsule (Compère 1990, Seidl *et al* 2012, Alagboso *et al* 2014). Interestingly, the epicuticle can considerably increase in thickness at cuticle regions with more specific functions like in the anterior edges of the tergites, edges of the joint head cuticle (Seidl *et al* 2011, Ruangchai *et al* 2013), in arthrodial membranes (figure 2(b)) (Ruangchai *et al* 2013), and the transparent cornea cuticle of the ommatidia in isopod compound eyes (figure 2(c)) (Alagboso *et al* 2014). At the edges of skeletal elements the

**Table 1.** Thicknesses of the outer and inner epicuticle as observed in different skeletal elements from the exoskeletons of various species of Isopoda and Decapoda.

Species	Structure	Outer epicuticle thickness/nm	Epicuticle thickness/nm	Author
<i>Ligia oceanica</i>	Complex eye cornea	50	350–800	Alagboso <i>et al</i> (2014)
<i>Ligia oceanica</i>	Head capsule	50	350	Alagboso <i>et al</i> (2014)
<i>Sphaeroma</i>	Complex eye cornea	20	200–800	Alagboso <i>et al</i> (2014)
<i>Porcellio scaber</i>	Pars incisiva	40	3000–10 000	Huber <i>et al</i> (2014)
<i>Porcellio scaber</i>	Joint head central	50	130–170	Ruangchai <i>et al</i> (2013)
<i>Porcellio scaber</i>	Joint head edge	50	1000	Ruangchai <i>et al</i> (2013)
<i>Porcellio scaber</i>	Membraneous layer	50	1200	Ruangchai <i>et al</i> (2013)
<i>Porcellio scaber</i>	Tergite	40	150	Seidl <i>et al</i> (2012)
<i>Titanetes albus</i>	Tergite	25–35	60–80	Hild <i>et al</i> (2009)
<i>Tylos europaeus</i>	Tergite	20–30	50–90	Seidl <i>et al</i> (2011)
<i>Tylos europaeus</i>	Tergite anterior edge	50	500–600	Seidl <i>et al</i> (2012)
<i>Oniscus asellus</i>	Tergite	40	240	Compère (1990)
<i>Ligidium germanicum</i>	Tergite	25	55	Vittori and Štrus (2014)
small <i>Trichonicidae</i>	Tergite	9–29	25–50	Vittori and Štrus (2014)
<i>Homarus americanus</i>	Claw, carapax		1000	Raabe <i>et al</i> (2006)
<i>Homarus americanus</i>	Joint condyles		4000	This work
<i>Homarus americanus</i>	Pars incisiva		40 000–400 000	This work
<i>Cancer pagurus</i>	Carapax	1000	3000–4000	Fabritius <i>et al</i> (2012)
<i>Cancer pagurus</i>	Pars incisiva		6000	This work



**Figure 2.** Structure of epicuticle from different skeletal elements. (a) Central region of *Porcellio scaber* joint head cuticle; arrowhead, waxy layer; ie, inner epicuticle; oe, outer epicuticle. (b) Epicuticle of arthrodistal membrane between the joint head cuticle and the coxal plates of *P. scaber*; white arrows, epicuticular canals. (c) Epicuticle of the transparent cuticle of ommatidia in the complex eye of *Ligia oceanica*; ee, epicuticular extensions. (d) Cleaved surface of native epicuticle of the pars incisiva in the mandibles of *P. scaber*. (e) Detail of (d) showing orthogonally oriented fibrils. (d) and (e) Altered from Huber *et al* (2014), copyright 2014 with permission from Elsevier. (f) and (g) Structure of epicuticle (epi) and the distal part of the exocuticle (exo) in the complementary sliding surfaces of hinge joints (inserts) connecting the pereopod segments of *Homarus americanus*.

increase of epicuticle thickness can be regarded as a transitional feature since epicuticle in the arthrodial membrane is also thick (Seidl *et al* 2011, Ruangchai *et al* 2013), likely to improve flexibility of the membranes. In the cornea thickening of the unmineralized epicuticle may attenuate the gradient in the optical density between the outer medium and the subjacent layer of calcite and thus reduce light reflection (Alagboso *et al* 2014). The thickest, up to 10  $\mu\text{m}$ , inner epicuticle was observed within the pars incisiva of the mandibles of isopods (Huber *et al* 2014, Huber *et al* 2015). The partes incisivae are the parts of the mandible that directly serve for cutting the food; in the case of *P. scaber* mostly leave litter. Here, the inner epicuticle likely serves mechanical functions. Therefore, it is of interest that the inner epicuticle differs in its structure from the epicuticle of the more general cuticle and of the transitions. Cleavage behavior of the inner epicuticle in the pars incisiva is anisotropic. In transversally cleaved samples the epicuticle appears composed of columns between 150 and 400 nm thick and with their long axes oriented perpendicular to the surface. Longitudinally cleaved samples result in box shaped structures, which edges are between 200 and 400 nm long (figure 2(d)). Surprisingly, the epicuticle of the pars incisiva contains fibrils with diameters between 10 and 14 nm (figure 2(e)). Their long axes are either oriented longitudinally, pointing towards the tip, or perpendicular to the surface of the pars incisiva. This orientation pattern of the fibrils, and possibly collapsed and therefore invisible epicuticular canaliculae are likely responsible for the anisotropic cleavage behavior. In light microscopic and TEM images the fibrils are indiscernible. Instead, an oblique striation can be observed that is likely due to the presence of the epicuticular canaliculae (Huber *et al* 2014).

At the proximal side of the inner epicuticle, the border to the exocuticle is often irregular or even forms extensions into the subjacent exocuticle. These extensions likely strengthen the transition between the two cuticular layers, thereby preventing delamination of the epicuticle. The extensions are usually short, like in tergites and the joint head (Seidl *et al* 2011, Ruangchai *et al* 2013). However, in the pars incisiva root-like epicuticular extensions are numerous and extremely long (Huber *et al* 2014). Because of its function and rather small diameter, its cuticle is expected to be exposed to substantial compressive, bending, and shearing forces that may promote delamination of the epicuticle. The long epicuticular extensions occur in a middle region starting about 100  $\mu\text{m}$  behind the tip of the pars incisiva, where the epicuticle is up to 10  $\mu\text{m}$  thick. From here, the length of the extensions tapers off with increasing diameter of the pars incisiva and decreasing thickness of the epicuticle. Near the base of the pars incisiva, where the epicuticle is still 4–5  $\mu\text{m}$  thick the extensions almost disappear. At the transition towards the base the thickness of the epicuticle

suddenly decreases from 4 to about 1.5  $\mu\text{m}$ . Between the tip and the base region, a typical exocuticle is lacking. The exocuticle with twisted chitin-protein fibrils begins within the base at the transition, filling the space between the thinning epicuticle and the endocuticle. Here the epicuticle forms an unusual hybrid material with the twisted stacks of the exocuticle (Huber *et al* 2014). Likely, this material serves to connect the epicuticle laterally with the exocuticle. In the cornea of the ommatidia of the compound eyes of *Ligia oceanica* the root-like epicuticular extensions appear specialized (figure 2(c)). They are longer in comparison to those of the surrounding head capsule and regularly distributed. The dimensions of the extensions and the spaces in between are way below the wavelength of light. Since the spaces between the extensions contain calcite, it appears possible that the extensions reduce a steep gradient in the refractive index between the epicuticle and the subjacent layer of calcite, thus improving the antireflective properties of the cornea cuticle (Alagboso *et al* 2014).

In the two decapod species we studied, the structural organization of the epicuticle differs significantly. In the lobster *Homarus americanus*, no clear distinction between an outer and an inner epicuticle was observed. The epicuticle's thickness is about 1  $\mu\text{m}$  in the cuticle forming the carapax, pereopods, and the uropod flippers. Vertical striations observed in fractured cuticle samples examined with SEM indicate the presence of many epicuticular canaliculae. In cuticle forming the sliding surfaces of hinge joints connecting the carpus and propodus in the claw, the thickness of the epicuticle increases to up to 4  $\mu\text{m}$  and it has a very smooth surface. Its cross section displays a vertical columnar structure consisting of numerous small canals filled with organic material. These canals provide a firm connection to the distal exocuticle that lacks mineral and is pervaded by numerous pore canals (figures 2(f), (g)). The thin epicuticle that covers the carapax and the claws of the crab *Cancer pagurus* contains no helicoidally arranged fibers and consists of a compact outer layer with a thickness of about 1  $\mu\text{m}$  and an inner layer with a thickness of 3–4  $\mu\text{m}$  pervaded by numerous pore canals (Hegdahl *et al* 1977, Fabritius *et al* 2012). The outer epicuticle consists mainly of epicuticular waxes, but the small pore canals of the inner layer contain mineral particles (Fabritius *et al* 2012).

### 2.1.2. Procuticle: exo-, endocuticle and membraneous layer

The exo- and endocuticle are the only layers in the isopod cuticle that contain mineral. In almost all skeletal elements, calcite occurs only in the exocuticle, sometimes together with ACC, whereas the endocuticle contains mineral in the form of ACC. The organic phase of both layers consists of chitin-protein fibrils or fibers that are generally, but not always, organized in the typical twisted plywood arrangement. Often the

cuticle contains another layer at the innermost (proximal) side that is called membraneous layer. This layer consists of twisted chitin protein fibrils as well, but contains per definition no mineral. In isopods this layer is sometimes present and sometimes lacking. There seems to be no correlation of its presence with species or skeletal element, suggesting that this layer is actually a part of the endocuticle that remained unmineralized. This interpretation is further supported by proximal partly mineralized stacks of the membraneous layer, of which some are continuous with mineralized stacks of the endocuticle (figure S1).

The endo- and exocuticle are structurally distinct in most species and skeletal elements; however, sometimes it is difficult to differentiate between these two layers. Historically, the border between the endocuticle and exocuticle is not very well defined. Nevertheless, in many species a clear decision can be made on the base of the structure and differences in TEM staining. In these the endocuticle appears smooth, branching of pore canals is rather rare, and staining is weak, whereas the exocuticle contains larger interconnecting pore canals that often branch and, in stained TEM sections, it appears darker than the endocuticle (figure 3(a)).

The exocuticle of isopods is subdivided in at least two layers of distinct structure and composition, the distal and proximal exocuticle. The distal exocuticle is characterized by thick fibers, in which several crystalline chitin fibrils are embedded in a proteinaceous matrix (figures 3(b) and (c)). These fibers form a twisted-plywood structure. The single fibers are spatially separated by comparatively long distances resulting in a rather low concentration of organic components within the layer. In contrast, the proximal exocuticle as well as the endocuticle is formed by single mineralized chitin-protein fibrils. These fibrils consist of a central core of a chitin filament surrounded by proteins (Carlström 1957, Blackwell and Weih 1980). This organic core is surrounded by mineral (figures 3(d)–(f)).

Distinct distal and proximal types of exocuticle occur in any isopod species and skeletal element we have studied so far, however, the relative thickness of the distal and proximal part of the exocuticle varies considerably between species. For the terrestrial isopod species there appears to be a systematic relationship between the thickness of the distal exocuticle and their mayor phylogenetic taxa. Species belonging to the suborder Tylidae have a very thick distal exocuticle (figures 4(a) and (b)) (Seidl and Ziegler 2011, Seidl *et al* 2011, Huber *et al* 2015) whereas those of the Ligiiidae and Crinochaeta have a thin layer of distal exocuticle (Seidl and Ziegler 2012, Ruangchai *et al* 2013, Alagboso *et al* 2014, Huber *et al* 2014). A distal type of exocuticle has been also found in the marine isopod species *Sphaeroma serratum* (Ansenne *et al* 1988, Alagboso *et al* 2014).

Similar to isopods, the exo- and endocuticle of the decapod species *H. americanus* and *C. pagurus* are mineralized and their organic phase is in most cases organized in the form of twisted plywood. In both species the proximal layer is constituted by an unmineralized membraneous layer consisting of twisting fibrous layers that is only present in the intermolt stage. Exo- and endocuticle are always structurally distinct and differ in the helical pitch of the twisted plywood, the type of mineralization and size and distribution of pore canals (Fabritius *et al* 2011). In general, the exocuticle has a uniform thickness in all skeletal elements forming the body shell, 200 to 300  $\mu\text{m}$  in *H. americanus* and about 300  $\mu\text{m}$  in *C. pagurus*. In contrast, the thickness of the endocuticle varies in both species between several millimeters in chelipeds down to about 40  $\mu\text{m}$  in e.g. the uropods, indicating that the overall mechanical stability of a load-bearing skeletal element is adjusted by the thickness of the endocuticle.

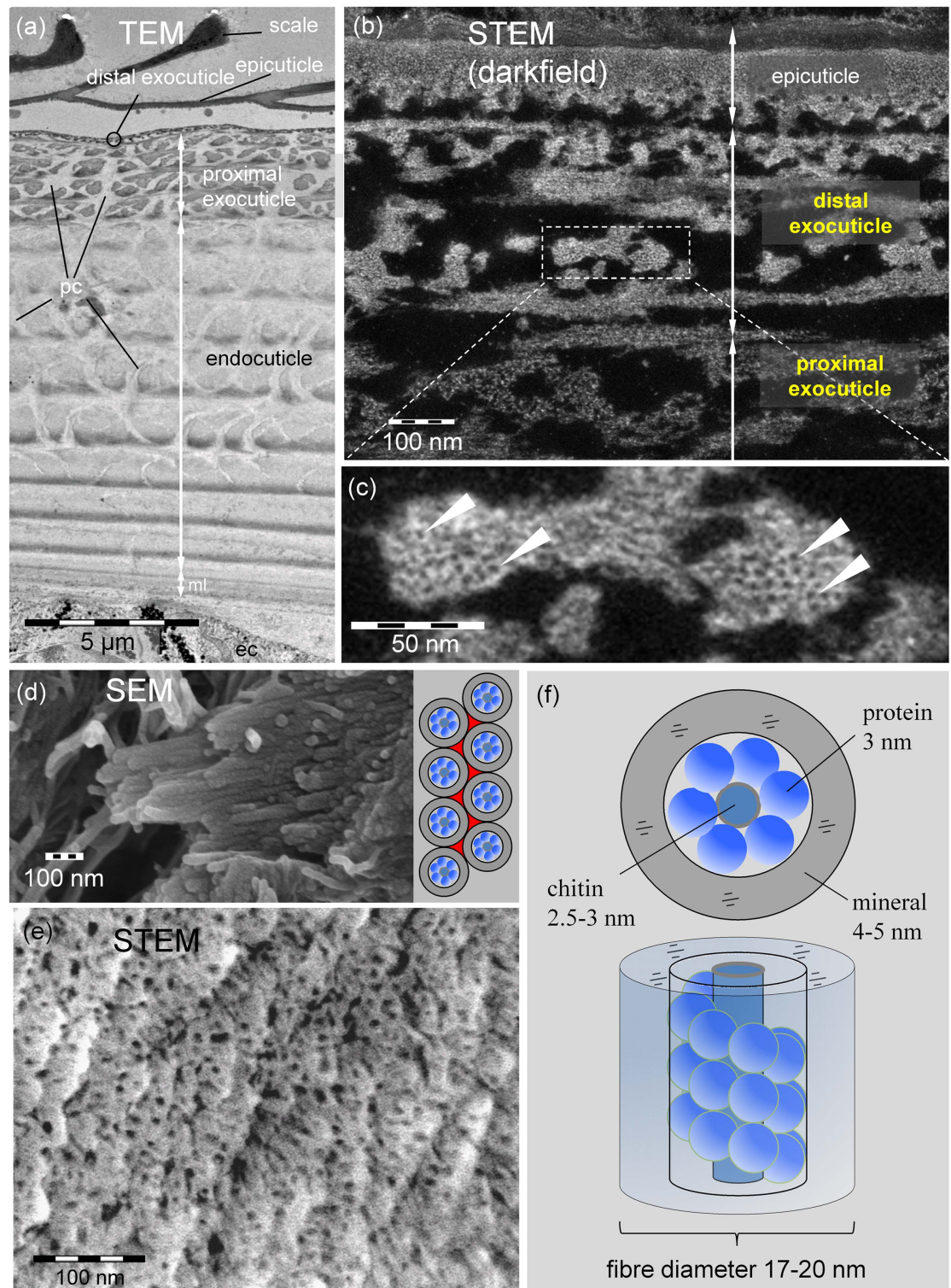
In *H. americanus* (see Raabe *et al* 2006), the exocuticle is structurally uniform and consists of uniformly thick twisted plywood layers formed by individual chitin nanofibrils surrounded by small, spherical mineral particles. In *C. pagurus* (see Fabritius *et al* 2012), the exocuticle forms three distinct layers that differ in the type of mineral phase. The stacking height defined by the helical pitch of the plywood structure increases from distal to proximal. The ultrastructure of the lobster's endocuticle is basically identical to that of the exocuticle and only differs in the stacking height which is much larger with about 30  $\mu\text{m}$  as compared to about 5  $\mu\text{m}$  in the exocuticle. In *C. pagurus*, the endocuticle is formed by large chitin-protein fibers with diameters of about 70 nm consisting of parallel arrays of 7 nm thick nanofibrils. Each fiber is surrounded by 20–50 nm thick mineral granules forming solid tubes. Compared to isopods, both decapod species possess a pronounced pore canal system in both layers.

### 3. Structural and compositional aspects of selected skeletal elements

#### 3.1. Exo- and endocuticle of the body shell

The dorsal cuticular segments of the pereopod and pleomeres (the tergites) form the major part of the protective cuticle of an isopod and must sustain endogenous loads brought about by muscle contractions and exogenous loads originating from predation. In Decapoda, the cephalothorax is protected by the carapax that forms the main part of the body shell. In the lobster, it is ventrally open and only loosely connected to the body segments it encloses, while in crabs it forms a solid shell that is firmly connected to the ventral plates carrying the appendages. The main function of this cuticle type is to provide mechanical stability against external and internal loads.

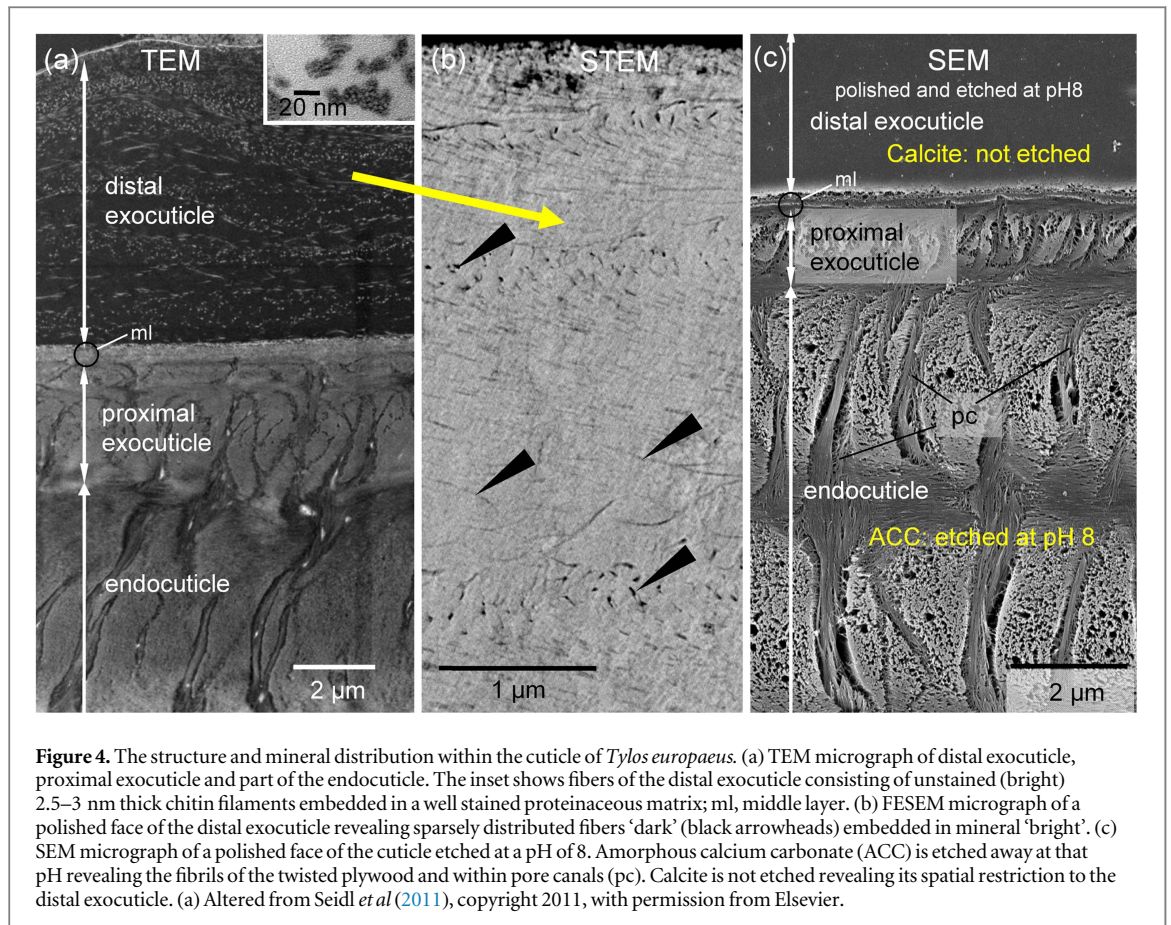




**Figure 3.** The general structure of isopod cuticle showing TEM (a), dark field STEM (b) and (c), FESEM (d) and STEM (e) micrographs. (a) Overview of tergite cuticle depicting epicuticular scales, distal and proximal exocuticle, endocuticle, membrane layer (ml) and pore canals (pc). (b) Micrographs of the distal exocuticle of *P. scaber* containing thick fibers. (c) Detail from (b) showing a cross section through a large fiber that consists of unstained chitin filaments with diameters of 2.5–3 nm (white arrowheads) surrounded by an intensely stained proteinaceous matrix. (d) Proximal exocuticle of *Tylos europaeus* showing individual rod like fibrils. Note that sharp triangular structures as depicted in red in the scheme on the right side of (d) cannot be observed. (e) Thin section through non-demineralized endocuticle of *T. europaeus*, organic fibrils (dark) surrounded individually by mineral (bright). (f) Scheme of proposed distribution of organic components and mineral within single mineralized fibrils. (a)–(c) Altered from Seidl and Ziegler (2012) CC BY, Copyright 2012, (d) and (e) altered from Seidl et al (2011), copyright 2011 with permission from Elsevier.

Consequently, structure and composition of the carapax and the rigid parts of the pereopods are largely the same, and only the cuticle thickness differs.

In general, the cuticle of skeletal elements such as chelipeds that are exposed to high mechanical stress is thicker than that of the carapax.



**Figure 4.** The structure and mineral distribution within the cuticle of *Tylos europaeus*. (a) TEM micrograph of distal exocuticle, proximal exocuticle and part of the endocuticle. The inset shows fibers of the distal exocuticle consisting of unstained (bright) 2.5–3 nm thick chitin filaments embedded in a well stained proteinaceous matrix; ml, middle layer. (b) FESEM micrograph of a polished face of the distal exocuticle revealing sparsely distributed fibers 'dark' (black arrowheads) embedded in mineral 'bright'. (c) SEM micrograph of a polished face of the cuticle etched at a pH of 8. Amorphous calcium carbonate (ACC) is etched away at that pH revealing the fibrils of the twisted plywood and within pore canals (pc). Calcite is not etched revealing its spatial restriction to the distal exocuticle. (a) Altered from Seidl *et al* (2011), copyright 2011, with permission from Elsevier.

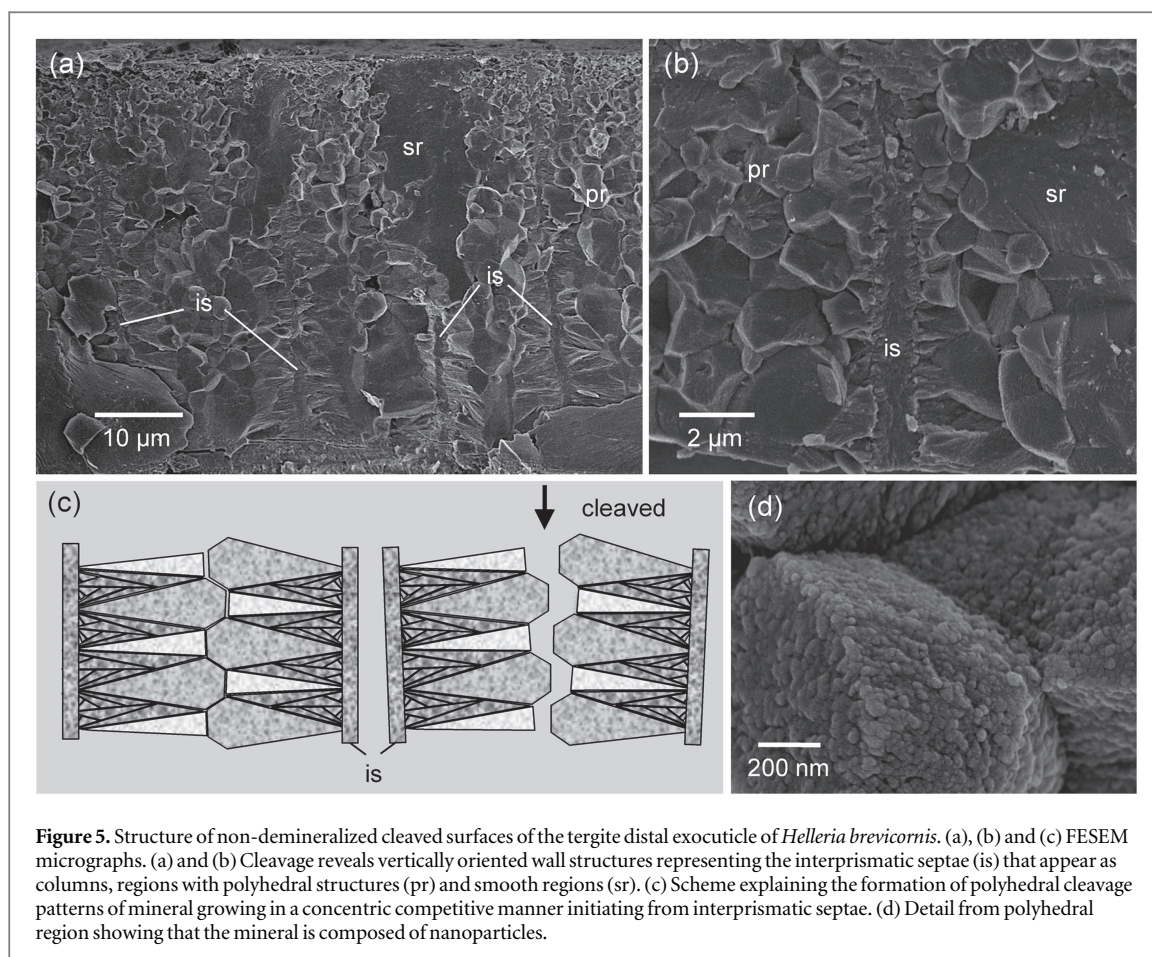
### 3.1.1. The exocuticle of tergites

Raman spectroscopic imaging (Seidl *et al* 2011) and dissolution experiments show that in tergites of *T. europaeus* the distal exocuticle is mineralized by calcite and virtually devoid of ACC, whereas the proximal exocuticle and a thin median layer that separates the distal from the proximal cuticle is mineralized by ACC (figure 4(c)). Besides calcium carbonate the exocuticle contains minor amounts of calcium phosphate as well. First Raman spectroscopic imaging (Seidl *et al* 2011) and later electron backscatter diffraction (EBSD) (Seidl *et al* 2012) have shown that the calcite layer consists of mineral grains that can be several tenths of  $\mu\text{m}$  in size. When the cuticle of *T. europaeus* or *H. brevicornis* is cleaved in its native non-demineralized state unusual polyhedral structures are revealed that are interspersed by smooth regions. In addition, the distal cuticle contains vertically oriented structures that appear column-like in transversally cleaved cuticle. From their margins, short fan-shaped structures radiate horizontally (Seidl *et al* 2011, Seidl and Ziegler 2011), (figures 5(a) and (b)). The scheme in figure 5(c) explains how the structure of cleaved faces may possibly be affected by mineral growth patterns. The vertical structures likely correspond spatially to the interprismatic septa that have been shown to be the calcification initiation sites in crab cuticle (Giraud-Guille 1984, Dillaman *et al* 2005). Smooth and polyhedral regions have a radial growth pattern of

20–30 nm sized granules suggesting crystallization from an amorphous precursor (figure 5(d)). Such a crystallization from amorphous precursors has been demonstrated for the tergite exocuticle of *P. scaber* (Neues *et al* 2011). It is of interest that the size of the polyhedral structures in *T. europaeus* of about 1–2  $\mu\text{m}$  is about one order of magnitude below the size of calcite grains of coherent crystallographic orientation. This suggests that crystallization spreads across several polyhedral structures after they have formed from ACC granules. The organic fibers within the distal exocuticle apparently have no effect on the structure of cleavage surfaces. In addition, there was no particular preferred angle between the crystallographic orientation of the calcite grains and fiber orientation, and no preference towards the horizontal or vertical direction. However, there is a weak *c*-axis orientation preference of around  $45^\circ$  towards the animal's longitudinal axis (Huber *et al* 2015).

In contrast to the distal exocuticle, in the proximal part of the exocuticle the orientation pattern of organic fibrils has a strong effect on the structure of the cleavage face. Cleavage occurs preferentially along the long axis of 20 nm thick fibrils resulting in a layered cleavage face following the twisted plywood.

In Crinochaeta like *P. scaber* and *A. vulgare*, and the Trichoniscidae *Titanethes albus* the distal exocuticle is generally very thin, around 500 nm, and the proximal exocuticle is thick, in *A. vulgare* several



**Figure 5.** Structure of non-demineralized cleaved surfaces of the tergite distal exocuticle of *Helleteria brevicornis*. (a), (b) and (c) FESEM micrographs. (a) and (b) Cleavage reveals vertically oriented wall structures representing the interprismatic septae (is) that appear as columns, regions with polyhedral structures (pr) and smooth regions (sr). (c) Scheme explaining the formation of polyhedral cleavage patterns of mineral growing in a concentric competitive manner initiating from interprismatic septae. (d) Detail from polyhedral region showing that the mineral is composed of nanoparticles.

tenths of  $\mu\text{m}$ . The proximal exocuticle has a vast system of large, sometimes interconnecting, pore canals (figure 3(a)). Calcite occurs within both, the distal and proximal exocuticle (Hild *et al* 2009, Seidl and Ziegler 2012, Seidl *et al* 2012). In *P. scaber* and *A. vulgare* ACC occurs within pore canals, however at proximal regions only (Seidl and Ziegler 2012). In the cave dwelling species *T. albus* the distal half, including part of the proximal exocuticle, contains calcite and ACC, whereas the remaining proximal layers of the exocuticle, including a distinct middle layer between the distal and proximal exocuticle, contain ACC only.

Cleaved faces through non-demineralized cuticle reveal that in all four species the distal exocuticle appears smooth, consisting of rows of 20–30 nm thick mineral granules (Hild *et al* 2008). In the proximal exocuticle of *P. scaber* and *A. vulgare* the fracture more often runs across a number of fibrils instead along their long axes, resulting in a blocky appearance of cleaved faces (figure 6(a)).

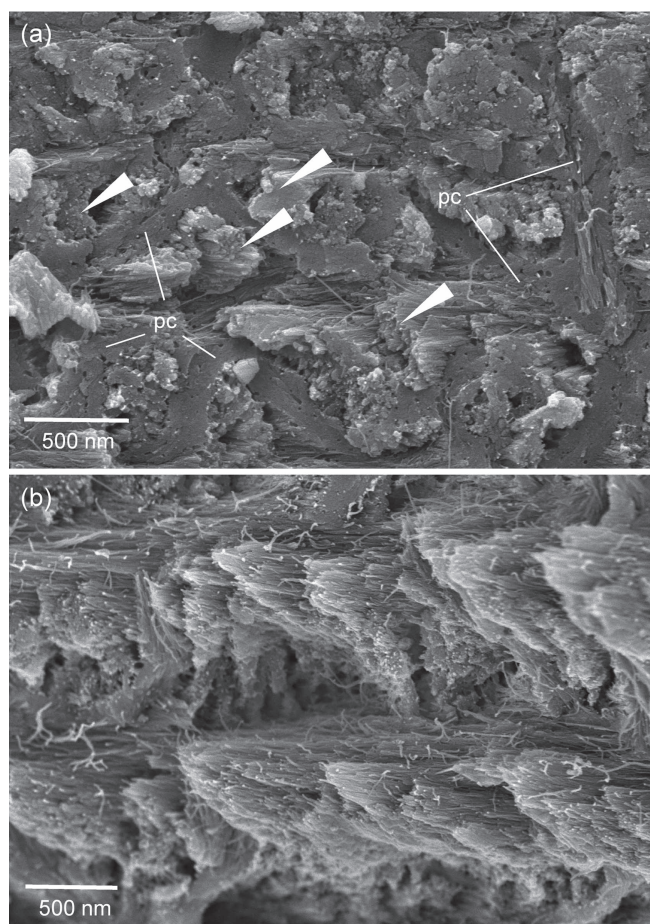
### 3.1.2. The endocuticle of tergites

The endocuticle of tergites is mineralized by ACC only. In *P. scaber* and *A. vulgare* there is a distinct difference in the fracture behavior of the non-demineralized endocuticle compared to that in the proximal exocuticle described above. In the endocuticle, cleavage through the cuticle occurs more frequently along the

longitudinal axis rather than across the fibers. This leads to a peculiar spiny appearance of cleaved surfaces resulting from alternating cone like protrusions of chitin-protein bundles and depressions of complementary shape (figure 6(b)). Since in both the proximal exocuticle and the endocuticle single fibrils are surrounded by mineral, the different cleavage behavior likely indicates differences in the strength of the lateral conjunction between the fibrils mineral phase.

### 3.1.3. Magnesium and phosphate content of tergite cuticle

EDX analysis shows that the mineral phase of the tergite cuticle contains small amounts of magnesium and considerable amounts of phosphorus. In *Tylos europaeus* the local molar magnesium/calcium ratio of about 10/100 is comparatively high, likely because the vicinity of the animals habitat to the marine environment, and thus ready access to the high magnesium content of seawater. In all species studied, there is only little local variation in the magnesium concentration, whereas the phosphorus content can vary between horizontal layers. In *P. scaber* and *A. vulgare*, highest phosphorus content was observed in regions of the endocuticle. In *T. europaeus* the thin middle layer between the distal and proximal exocuticle has a higher molar phosphorus/calcium ratio of 3–4/100 in comparison to the otherwise low ratio



**Figure 6.** FESEM micrographs from surfaces of non-demineralized cleaved tergite cuticle of *Porcellio scaber*. (a) In the proximal exocuticle cleavage occurs predominantly across the fibrils (white arrowheads) resulting in a 'blocky' appearance; pc, pore canals containing mineral. (b) In the endocuticle cleavage takes place predominantly along the fibrils. This results in cone like protrusions of chitin-protein bundles and depressions of complementary shape, together leading to a 'spiny' appearance.

below 1/100 (Hild *et al* 2008, Hild *et al* 2009, Seidl *et al* 2011).

Atomic absorption spectroscopy (AAS) has shown that for *P. scaber* and *A. vulgare* the molar magnesium/calcium ratio of 0,048 and 0,040 is somewhat lower than the value of 0.057 in *T. europaeus* (Becker *et al* 2005, Seidl *et al* 2011). The lowest molar magnesium/calcium ratio of about 0.011 was found in the cuticle of the karst cave dwelling species *T. albus* (Hild *et al* 2009). For *T. europaeus* EDX analysis has shown that the magnesium/calcium ratio can locally be as high as 0.1 (Seidl *et al* 2011). Quantitative synchrotron radiation based XRD analysis of tergite cuticle heated to 1000 °C revealed the presence of hydroxyapatite that cannot be detected if heat treatment was omitted. Combined with thermogravimetry and AAS these results indicate the presence of ACP in the native tergite cuticle, which amounts to 18%, 13%, 11% and 7.5% of total tergite mineral content in *P. scaber*, *A. vulgare*, *T. albus* and *T. europaeus*, respectively (Becker *et al* 2005, Neues *et al* 2007, Hild *et al* 2009, Seidl *et al* 2011).

In the cuticle, phosphate may either occur within phosphorylated proteins or as calcium phosphate. The

high concentrations of ACP indicate that phosphorus within the tergite cuticle occurs mainly in the mineral rather than the organic phase. It has been shown that phosphate can stabilize the ACC phase (Bachra *et al* 1963, Reddy 1977). Therefore, higher phosphorus content within the endocuticle of *P. scaber* and *A. vulgare*, and within the middle layer between the distal and proximal exocuticle in *T. europaeus* suggests that phosphate may play a role in the stabilisation of the ACC phase. However, *in vitro* experiments that have been conducted in the presence of the organic matrix of the sternal ACC deposits, have shown that high concentrations of phosphate are not required for ACC formation and stabilisation (Hennig *et al* 2012, Habracken *et al* 2015).

### 3.1.4. The exo- and endocuticle of the decapod *Homarus americanus*

A characteristic deviation from the general crustacean cuticle model observed in load-bearing cuticle of the lobster *H. americanus* is that the chitin-protein fiber level (see figure 1) is absent. Instead, the higher structural levels of both the mineralized exo- and endocuticle consist of chitin nanofibrils only

(Fabritius *et al* 2011). In both layers, the mineral phase consists of 10 to 30 nm thick spherical particles separating the nanofibrils. Despite this structural similarity, the mineral phases in exo- and endocuticle differ. Similar to isopod species, calcite is mainly located within the exocuticle (Al-Sawalmih *et al* 2008), but it is not homogeneously present. Instead, column-shaped calcitic regions are separated by roughly equal-shaped regions containing larger amounts of phosphates, as revealed by confocal Raman microscopy (Kunkel *et al* 2012, Fabritius unpublished). In the endocuticle, the mineral phase consists of ACC (Raabe *et al* 2006, Al-Sawalmih *et al* 2008). The large pore canals present in the endocuticle generate a honeycomb-like structure (Raabe *et al* 2005, 2006, Romano *et al* 2007) with anisotropic deformation behavior. In contrast to classical honeycomb structures, the twisted plywood arrangement of the fibrils makes the in-plane direction stiffer than the normal direction of the cuticle. Flexible tubes located in the pore canals further increase fracture resistance in transverse direction (Sachs *et al* 2006a, 2008). The combination of twisted plywood and honeycomb architecture of lobster cuticle results in optimized mechanical properties independent of the direction of imposed stresses. Due to their large size, the pore canals in lobster cuticle are not only a transport system, but also help to reduce weight and thus lower the energetic cost of mineralization for this large species.

### 3.1.5. The exo- and endocuticle of the decapod *Cancer pagurus*

The most distinct structural characteristic of the cuticle forming the dorsal carapace of *C. pagurus* is that the exocuticle is pervaded by parts of the endocuticle that bulge out in distal direction forming concentrically arranged plywood (Fabritius *et al* 2012) that reminds of the structure of osteons in vertebrate bones (Weiner and Wagner 1998). Therefore, the exocuticle assumes two different types of macroscopic structure: regions where it comprises one fourth of the entire cuticle thickness and extremely thin regions with a significant reduction in stacking height where it covers the endocuticular bulge structures. From distal to proximal, the thick exocuticle can be subdivided into three regions with different microstructure and mineral composition. The distal region is heavily mineralized with magnesium-rich calcite particles that fill up most of the pore canals. The region below contains calcitic vertical extensions, the interprismatic areas (Drach 1939), that ensure a firm connection between the two regions. The main mineral in this region is rich in phosphate and most probably a  $\text{Mg-PO}_4\text{-CO}_3$ -phase (Fabritius *et al* 2012). The most proximal region of the exocuticle contains only little phosphate in the form of hydroxyapatite, the main mineral phase is ACC (Fabritius *et al* 2012). The mineral particles in both inner regions are nanoscopic and interspersed between individual chitin nanofibrils

as observed in exo- and endocuticle of the lobster (Raabe *et al* 2006, Fabritius *et al* 2009). The entire exocuticle is rich in carotenes. These differences in mineralization are directly reflected in the local mechanical properties with the outermost calcitic layer showing the highest stiffness and hardness followed by the proximal ACC-rich layer and the least stiff and hard central phosphate layer. The tubular mineralization mode of the chitin-protein fibers is present in the entire endocuticle. However, the mineral phase differs in regions forming the bulges, which contain magnesian calcite and regions located under the exocuticle, which are mineralized with ACC (Boßelmann *et al* 2007, Fabritius *et al* 2012). Surprisingly, the local stiffness and hardness are very uniform in the endocuticle despite the different mineral phases. In cuticle of *H. americanus*, regions mineralized with calcite have significantly better mechanical properties than regions containing ACC only (Sachs *et al* 2006b). This suggests that local differences in the distribution density of the organic fibers caused by the different stacking heights of the plywood also strongly influence the local mechanical properties. The centers of the bulges are extremely stiff and hard due to a high grade of mineralization together with low organic material content and the absence of open pore canals. The pore canals in the endocuticle resemble those of *H. americanus* in shape and size, but their edges are reinforced with mineralized fibers over their entire length. This reinforcement is suitable to enhance the resistance of the cuticle against compressive loads in normal direction and in transverse direction by preventing the pore canals to collapse. The organization of the cuticle forming the dorsal carapace of the crab *C. pagurus* suggests a high resistance against mechanical loads. The alternation of the extremely hard bulges and the calcite-containing distal layers of exocuticle on the dorsal surface of the carapace of *C. pagurus* provide an effective barrier against penetration by predators. The bulges can act like pillars that transfer compressive loads to lower regions of the endocuticle, where the transition from vertical to horizontal arrangement of the plywood layers can dissipate the energy in lateral direction. In addition, the many interfaces created by the differently structured and mineralized areas are ideally suited to guide, deflect and trap cracks initiated at the surface (Fabritius *et al* 2012).

### 3.2. Exo- and endocuticle of eyes

In compound eyes of isopods the cuticle forms arrays of microlenses. Like in tergite cuticle exo- and endocuticle are mineralized and contain fibrils forming a twisted plywood structure. However, because of its dual function, the structure and composition of the cornea's exo- and endocuticle has to compromise between properties as a mechanically protective shield and those of a light conducting material. This has been

studied in two isopod species: the marine isopod *Sphaeroma serratum* and the terrestrial species *Ligia oceanica* (Alagboso *et al* 2014). Both have many ommatidia and thus vision is an important sensory input for these species. Since the size of the pore canals cross section is within the wavelength scale of visible light they would lead to scattering of light affecting transparency of the cornea. Therefore, pore canals are lacking in the central region of the cornea. Because of their important mechanical function, pore canals are not completely avoided but restricted to the inter-ommatidial spaces within complex eyes. Furthermore, for *S. serratum*, polarized light microscopy shows that calcite crystals within the head capsule surrounding the eye are small causing light scattering and thus an opaque appearance of the cuticle. In the cornea cuticle, however, crystal size is much larger, thus increasing transmission of light. Furthermore, carotenoids that pigment the general cuticle in both species are lacking in the cornea cuticle.

Like in tergite cuticle calcite occurs in distal regions of the exocuticle, while proximal ones are mineralized by ACC. It is of interest that in the compound eye the thickness of the calcite layer decreases from the cuticle of the surrounding head capsule from 2 to 1  $\mu\text{m}$  in *L. oceanica* and from 12 to 4  $\mu\text{m}$  in *S. serratum*. In *S. serratum* the calcite containing distal exocuticle becomes thinner together with the calcite layer, whereas in *L. oceanica* the thickness of the exocuticle is maintained and only part of the proximal exocuticle becomes mineralized by calcite. It is unknown why thinning of the calcite layer is required in the cornea cuticle. Possibly, this has to do with the birefringence property of calcite, that is lacking in ACC and that would lead to high blur of transmitted light in too thick calcite layers.

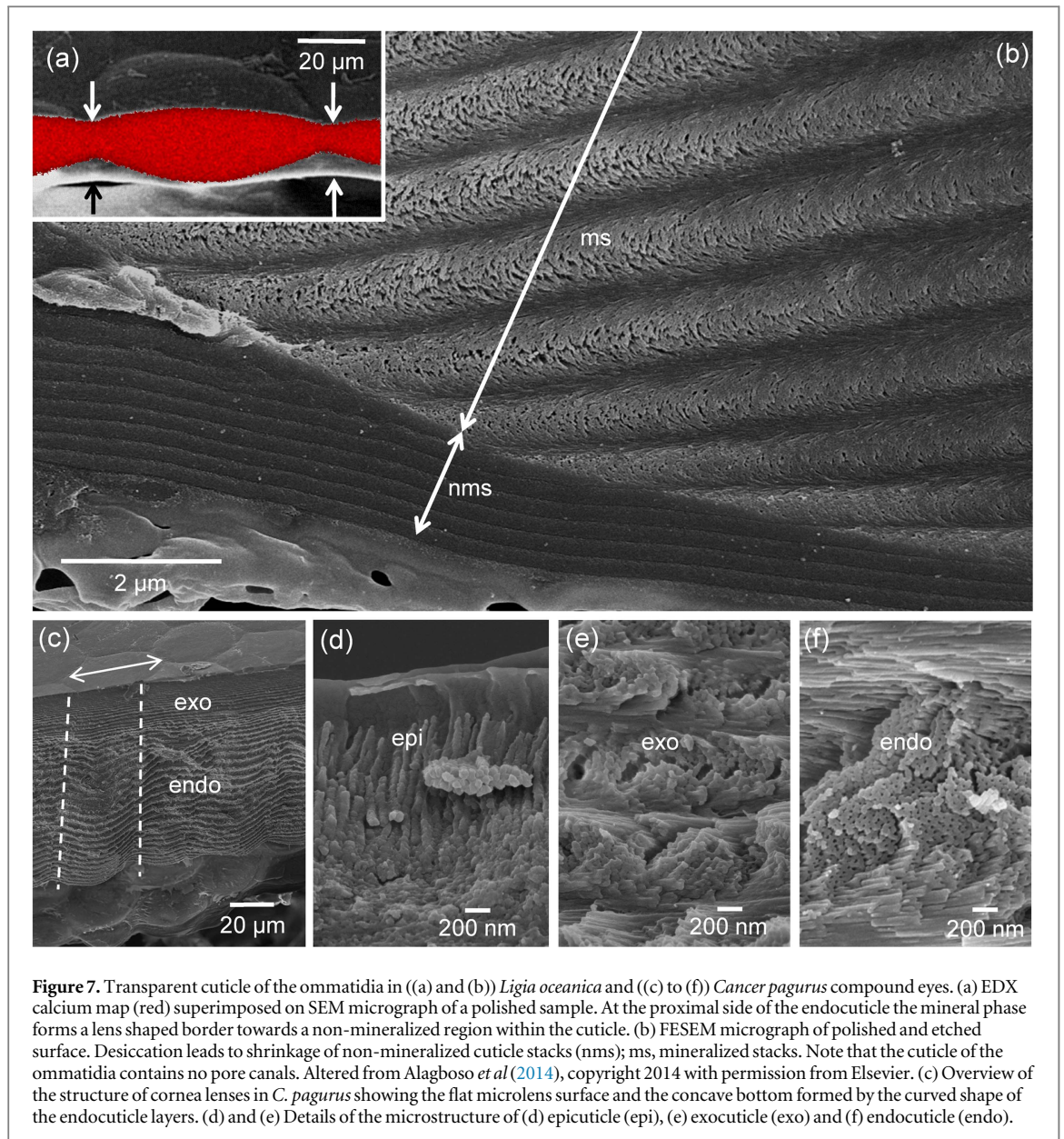
In addition of being transparent, the cornea functions as optical lenses as part of the dioptric apparatus of the ommatidia. This is reflected in the shape of the corneal lenses that is adapted to the habitat of the animal. In the aquatic species the curvature is much higher than in the terrestrial isopods, reflecting the differences in the refractive index of seawater and air, respectively. Both species form the lens shape within the cornea by thinning of the interommatidial spaces rather than thickening of the central region. However, thinning implies that the mechanical strength of the cornea cuticle is reduced. For *L. oceanica*, cuticle thickness appears to be a limiting factor to develop corneal lenses of high convexity. This may have led to the evolution of the differentially mineralized cornea cuticle, in which at the proximal side of the endocuticle the mineral phase forms a lens shaped border towards an un-mineralized region within the cuticle (figures 7(a) and (b)). That way the refractive power of the lens is increased and mechanical stability between the ommatidia is provided by organic material. In *S. serratum*, cuticle thickness is not limiting and such a differential mineralization is lacking.

The cuticle forming the cuticular cornea in the ommatidia (facets) of the compound eyes in the decapod *C. pagurus* is characterized by a significant decrease in thickness from about 600  $\mu\text{m}$  in the eye-stalk to about 100  $\mu\text{m}$  in the microlens area. This decrease is mainly due to a decrease in the stacking height of the plywood layers forming the endocuticle. In contrast to the corneal lenses of isopods, the distal surface of the ommatidia in *C. pagurus* is flat, and the proximal corresponding concave lens-shape is formed by the mineralized endocuticle alone (figure 7(c)). The ultrastructure of mineralized fibers in exo- (figure 7(e)) and endocuticle (figure 7(f)) corresponds to that of the surrounding load-bearing cuticle, but the epicuticle is thicker (figure 7(d)).

### 3.3. Exo- and endocuticle of joint heads in the appendages of Isopoda

Function-related modifications of cuticle structure and composition occur in the joint heads of *P. scaber* that connect the basis of the walking legs (pereopods) to the coxal plates at the body. Local variations occur between a central region and an edge region of the joint head. Most features within the central region resemble those in the tergites, with the exception that some islets of calcite can be found in the exocuticle. Another difference occurs in regard to the distribution of the 'spiny' and 'blocky' appearance in cleaved faces of non-demineralized cuticle (compare figure 6 for tergites). With the exception of the one or two stacks underneath the distal cuticle this distribution is reversed in the joint head. This is in agreement with the interpretation that this structural difference reflects differences in the strength of the connection between mineral surrounding the single fibrils, rather than differences in the organization of the organic fibrils.

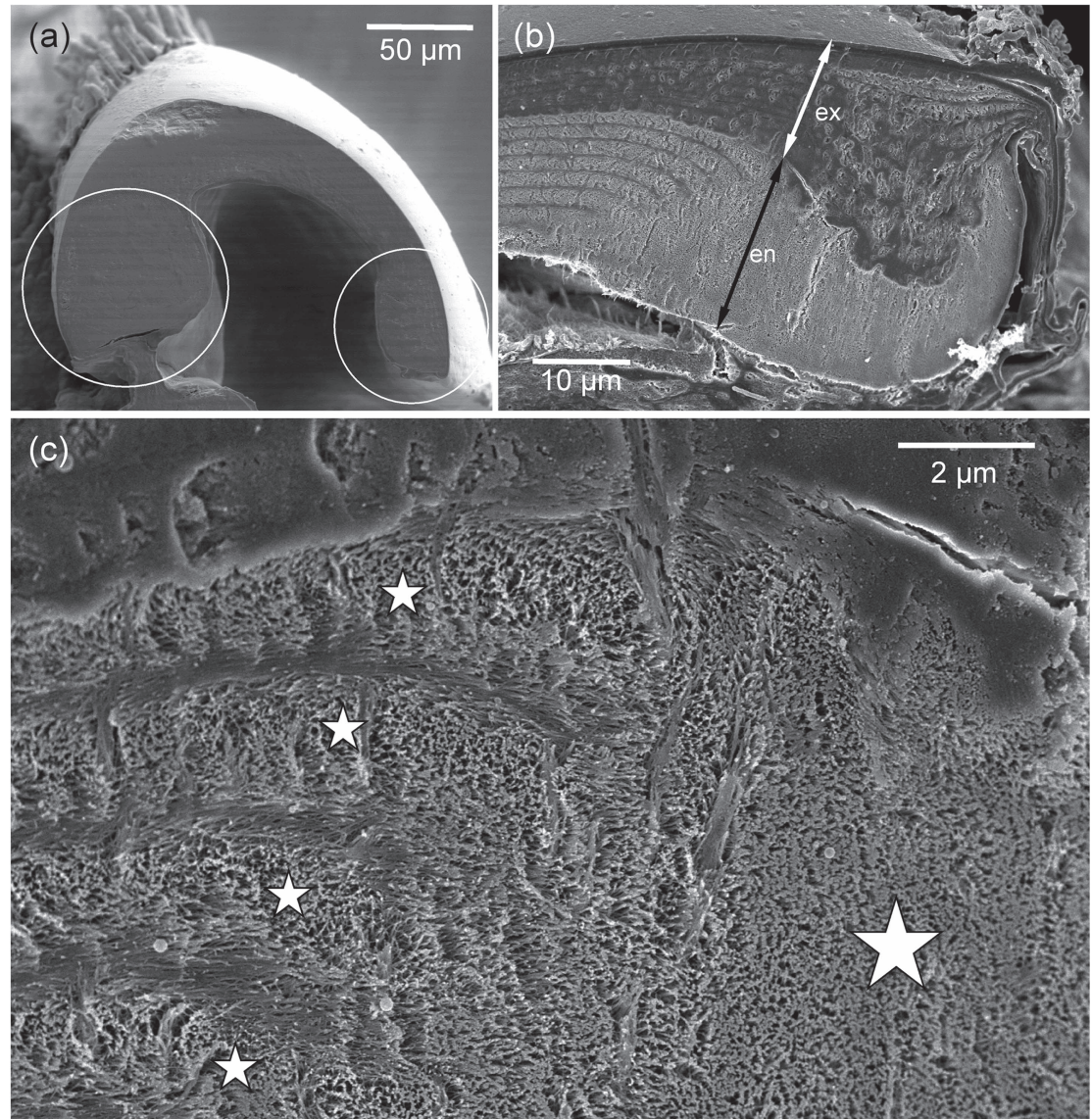
The structure and composition of the edge region suggests that it serves both, the stabilisation of the shape of the joint head and the transition to the flexible arthroal membrane. Furthermore, the edge provides the site of insertions of cuticular tendons for the extensor muscles of the legs. At the edge the cuticle doubles in thickness forming a bulge. The increase is brought about by thickening of the epicuticle and exocuticle (figures 8(a) and (b)). Likely, this feature serves to prevent deformation of the central region. The central region has a smooth outer surface that lies tightly apposed to its socket. Thus deformation of the cuticle of the central region would hinder smooth gliding within its socket during movement of the leg. The most striking feature of the edge region is the structural change of the endocuticle switching from a twisted plywood to an organization in which all fibrils run in parallel (figures 8(a) and (c)). The orientation of fibrils within the cuticle has a large effect on its mechanical properties (Vincent and Wegst 2004, Nikolov *et al* 2010). In comparison to cuticle of a



twisted plywood organization, stiffness increases by a factor of three in cuticle of unidirectional fibril orientation when forces are applied in parallel to the fiber direction (Vincent and Wegst 2004). Since during contraction of the extensor muscles forces are applied more or less in the direction of the endocuticular fibrils, their parallel orientation likely helps to further stiffen the bulgy edge region. *P. scaber* can run quite fast, e.g. when it tries to escape from a predator. Therefore, it is of interest that in slowly walking terrestrial isopods such parallel orientation of the fibrils is absent, supporting the function in stabilisation of the central region in *P. scaber* joint heads (Ruangchai *et al* 2013).

The edges of skeletal elements are the sites where the cuticle transforms from a stiff material to the flexible, at least in *P. scaber*, un-mineralized cuticle of the arthrodistal membranes. In the transition from the

edge to the arthrodistal membrane the thickness of the epicuticle increases at cost of the distal exocuticle that disappears at the very edges together with the endocuticle. Here, chitin-protein fibers project into the epicuticle forming a new type of cuticular layer that integrates structural characteristics of both epi- and exocuticle. The endo- and exocuticle at the transition is characterized by a particularly high content of phosphorus increasing from a phosphorus/calcium ratio between 0.06 and 0.13 in the central region to up to 0.22 at the transition to the arthrodistal membrane. Raman spectroscopic imaging has shown that calcite present in the exocuticle of the central region ceases at the beginning of the edge region and is replaced mostly by an ACC phase that co-localizes with phosphate, suggesting the presence of high amounts of ACP. It has been shown that the mineral type and phase (e.g. calcium carbonate versus



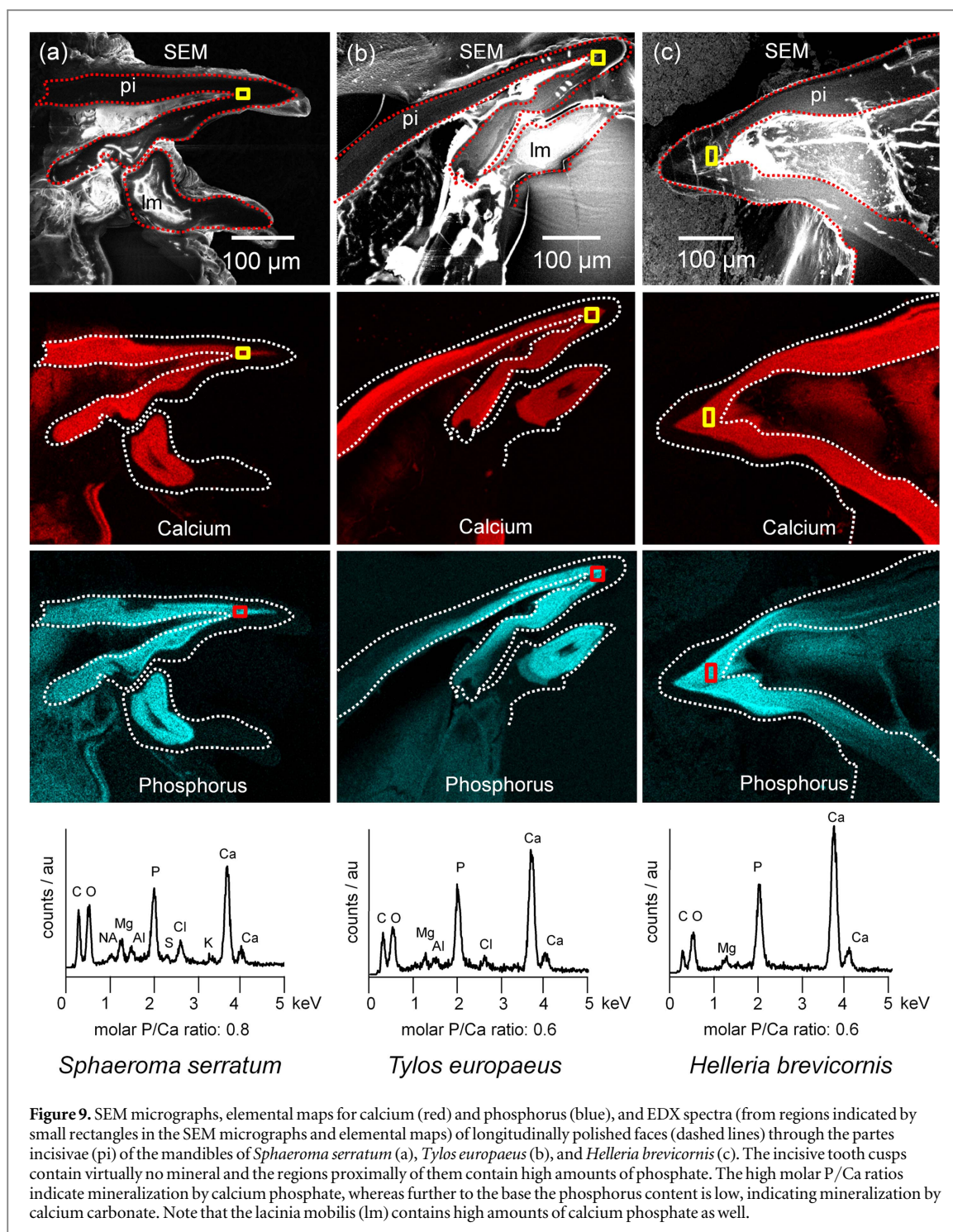
**Figure 8.** SEM (a) and FESEM (b), (c) micrographs of the joint head cuticle of the leg basis of *Porcellio scaber* that connects the leg with the coxal plate. (a) Transversally polished face through a joint-head revealing thickening of the cuticle (circles) at its edges. (b) Thickening of cuticle is brought about by an increase in the thickness of exocuticular stacks (ex). The endocuticle (en) decreases in thickness and the twisted plywood is replaced by unidirectional fibrils, which long axes are oriented along the edge. (c) Detail from (b), showing the transition between twisted plywood (small asterisks) and unidirectional fibrils (large asterisk). (b) and (c) altered from Ruangchai *et al* (2013), copyright 2013 with permission from Elsevier.

calcium phosphate and crystalline versus amorphous) and, if co-localized, their relative concentrations can result in very different mechanical properties that also depend on the cuticle's structural organization (Currey *et al* 1982, Bentov *et al* 2012, Fabritius *et al* 2012, Huber *et al* 2014). In mandibles of Isopoda, cuticle mineralized by ACP is less stiff and less hard than cuticle mineralized by ACC (Huber *et al* 2014). Likely the high ACP concentration, together with the high content of organic material and the integration of characteristics of epicuticle into the joint's exocuticle, makes the material in the transition more compliant and thus more resistant against crack formation during frequent changes of loads, caused by repeated contractions of extensors during walking and running.

### 3.4. Exo- and endocuticle of partes incisivea of mandibles

The main functional elements of mandibles involved in food processing are the incisor and molar processes (Manton and Harding 1964, McLaughlin 1980, Richter *et al* 2002), also termed pars incisive (PI) and pars molaris (PM). The PI's of the mandibles carry the cutting edges, these are very thin and the forces for cutting are exerted by the muscles at and within the large corpus of the mandibles. These muscles are the largest of the isopod body (Schmalzfuss 1974) indicating that mechanical loads on the PI must be highest among the isopod skeletal elements. Although one would expect that the tip of the pars incisiva would be the hardest part of the PI, this is not the case. The tip is not mineralized at all in the terrestrial species *P. scaber*,

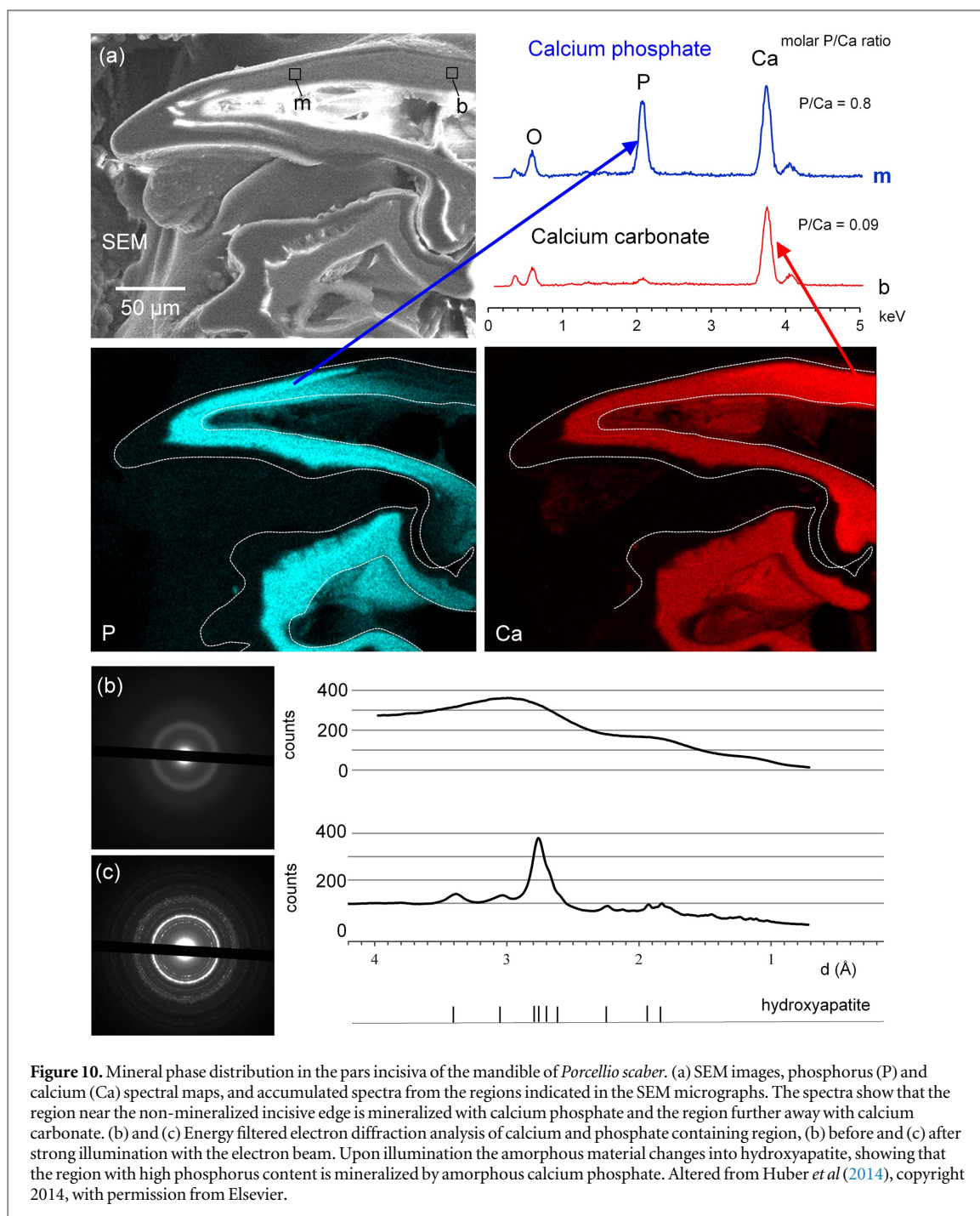




**Figure 9.** SEM micrographs, elemental maps for calcium (red) and phosphorus (blue), and EDX spectra (from regions indicated by small rectangles in the SEM micrographs and elemental maps) of longitudinally polished faces (dashed lines) through the partes incisivae (pi) of the mandibles of *Sphaeroma serratum* (a), *Tylos europaeus* (b), and *Helleria brevicornis* (c). The incisive tooth cusps contain virtually no mineral and the regions proximally of them contain high amounts of phosphate. The high molar P/Ca ratios indicate mineralization by calcium phosphate, whereas further to the base the phosphorus content is low, indicating mineralization by calcium carbonate. Note that the lacinia mobilis (lm) contains high amounts of calcium phosphate as well.

*T. europaeus* (Huber et al 2015), *H. brevicornis* and the marine isopod *S. serratum* (figure 9). However, the PI is strongly melanised reminding to the PI of insects that are strongly sclerotized and melanized as well. In the tip region, the cuticle underneath its thick epicuticle forms two layers and a central core. The most distal one of these, layer 2, consists of many sheets oriented perpendicular to the surface and longitudinally to the teeth cusps. They consist of granular material and 12–15 nm thick fibrils oriented towards the tip. Proximally these sheets turn, forming layer 3, in which the sheets planes are oriented transversally

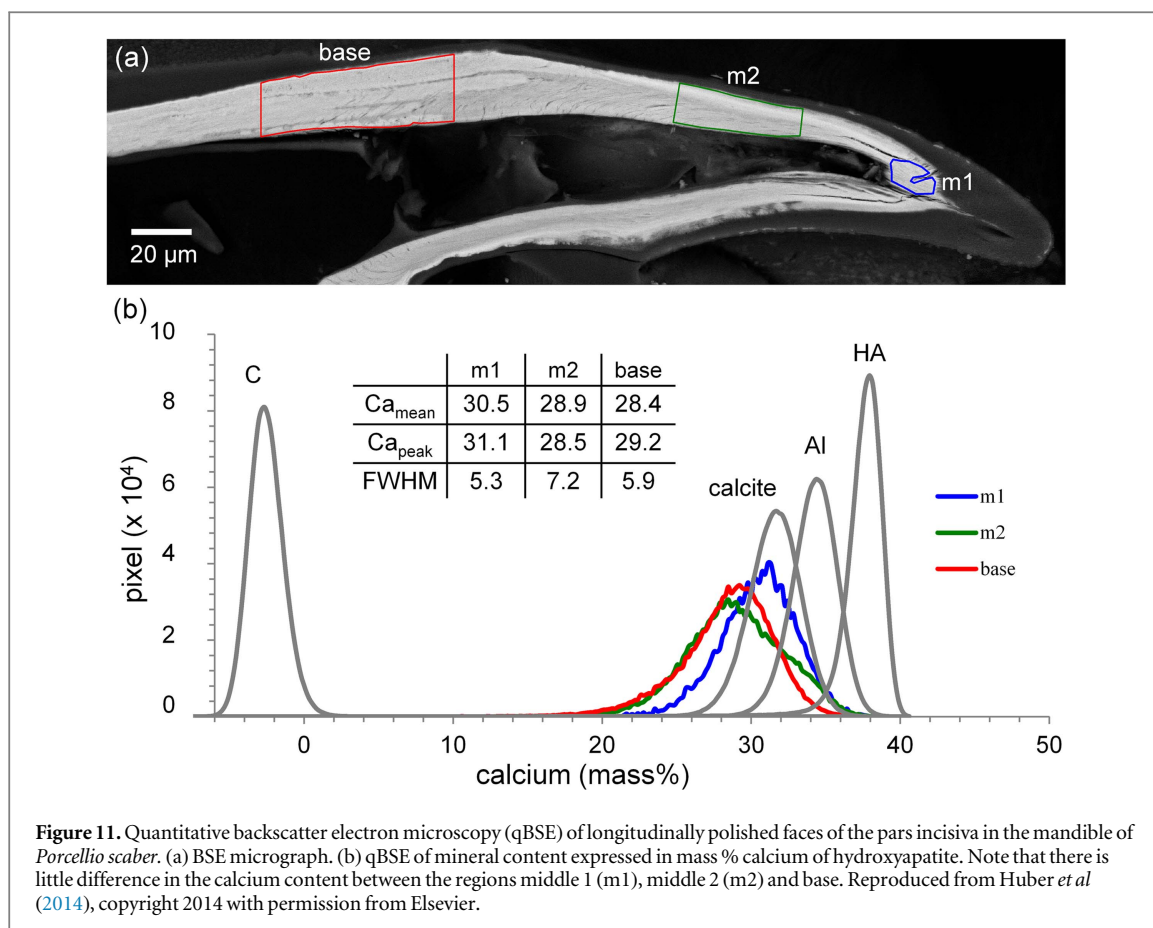
and filaments are oriented circumferentially. The central core consists of 12–15 nm thick densely packed fibrils oriented towards the cutting edge. In the middle region of the PI a typical exocuticle is lacking, so that the endocuticle begins just underneath the thick epicuticle. The fibrils of the endocuticle have a diameter of about 20 nm. Underneath the region with epicuticular extensions, fibrils form an about 15  $\mu\text{m}$  thick layer in which fibrils are oriented in parallel with their long axes pointing towards the cutting edge of the PI. Only in an about 2  $\mu\text{m}$  thick proximal layer the fibrils form a twisted plywood.



**Figure 10.** Mineral phase distribution in the pars incisiva of the mandible of *Porcellio scaber*. (a) SEM images, phosphorus (P) and calcium (Ca) spectral maps, and accumulated spectra from the regions indicated in the SEM micrographs. The spectra show that the region near the non-mineralized incisive edge is mineralized with calcium phosphate and the region further away with calcium carbonate. (b) and (c) Energy filtered electron diffraction analysis of calcium and phosphate containing region, (b) before and (c) after strong illumination with the electron beam. Upon illumination the amorphous material changes into hydroxyapatite, showing that the region with high phosphorus content is mineralized by amorphous calcium phosphate. Altered from Huber *et al* (2014), copyright 2014, with permission from Elsevier.

Most interestingly, the mineral phase in the middle region of the PI has a high phosphorus/calcium ratio in all four species studied (figures 9 and 10(a)). In *P. scaber* the ratio is 0.8 near the tip that gradually decreases to 0.09 near the base of the PI (figure 10(a)). Electron spectroscopic diffraction analysis shows that the mineral has an amorphous state and crystallizes to hydroxyapatite upon strong irradiation with the electron beam (figures 10(b) and (c)). A classical exocuticle with its distinct distal and proximal layers occurs within the base of the PI and the corpus of the mandible. EDX and EBSD analysis shows that the base is mineralized mainly by calcium carbonate with calcite occurring mostly in the exocuticle and ACC within the

endocuticle. The differences in fibril orientation preference and mineralization greatly affect the local stiffness and hardness in the PI cuticle. Nanoindentation experiments (Huber *et al* 2014) have shown that higher values are recorded from transversal and lower values from longitudinally oriented polished planes through the PI. This reflects the dominance of fibril orientation towards the tip of the PI, since chitin fibrils are much stiffer in the longitudinal direction (Nikolov *et al* 2010). This direction corresponds to that of highest mechanical load during muscle contraction, indicating that fiber orientation is adapted to the function of the PI. Furthermore, the reduced elastic modulus and hardness increase from the tip to the base of the

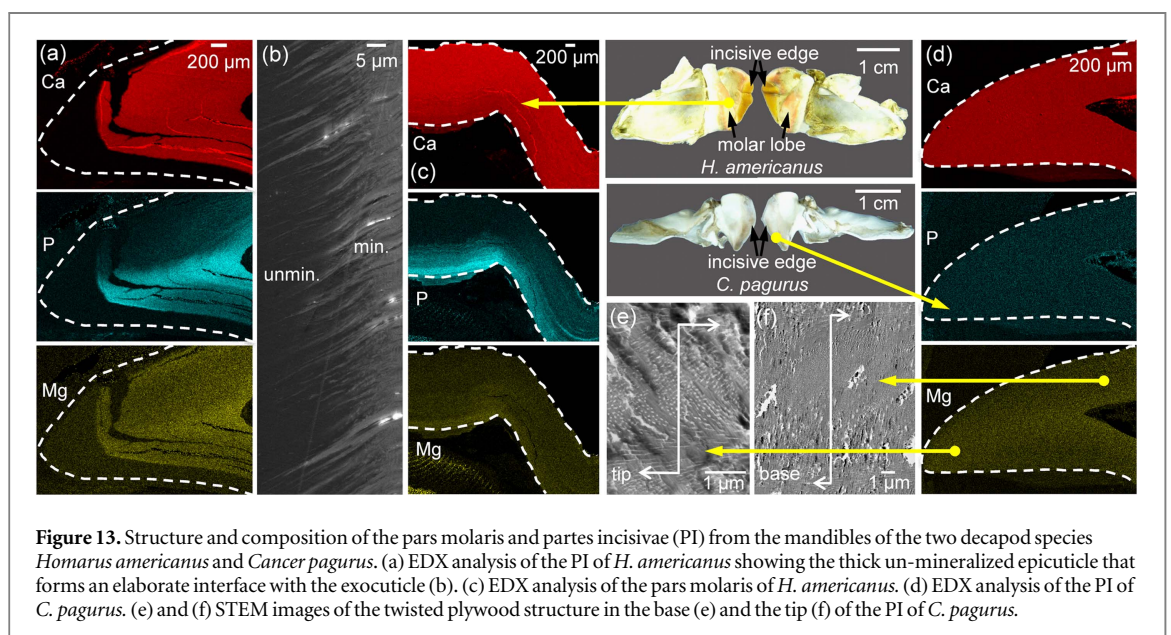
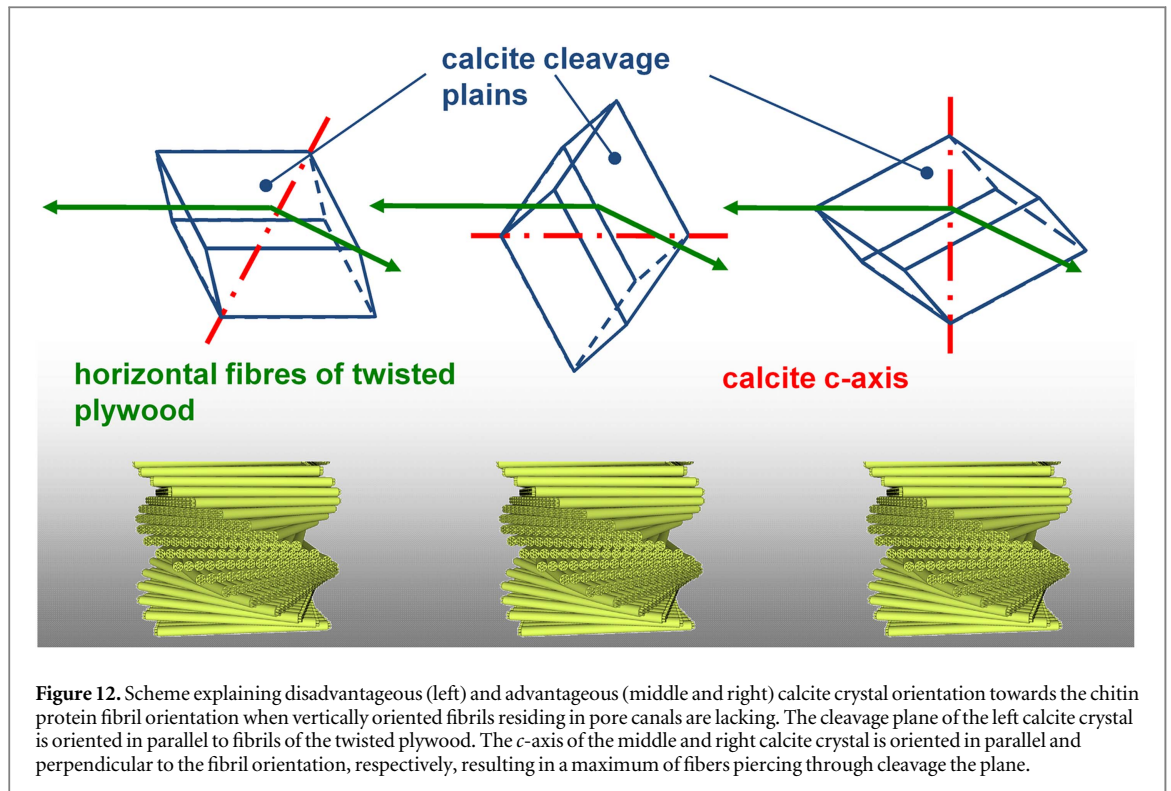


PI. Since a quantitative backscatter electron analysis indicates no large change in the degree of mineralization from the middle region to the base (figure 11), the corresponding increases must be due to the replacement of ACP by ACC. The graded increase in stiffness towards the base of the PI is accompanied by an increase in cross-section area. Thus mechanical load onto the cuticle material is higher at the tip than in the base of the PI and the large main corpus of the mandible. Likely the higher compliance, caused by the unusual mineralization with ACP instead of ACC, helps to avoid failure of the cuticle caused by transversal components of the load when high forces are conveyed from the teeth cusps to the corpus of the mandible.

Within the base of the PI and the corpus of the mandibles of *T. europaeus*, calcite is restricted to the distal type of exocuticle, just like in tergites of the species. EBSD analysis of calcite within the PI of *P. scaber* and *T. europaeus* revealed that the calcite crystal orientation distribution has species-specific differences that appear to be connected to the differences of the organization of the organic matrix (Huber *et al* 2015). In *P. scaber* the calcite layer is dominated by the proximal type of exocuticle that has a dense distribution of horizontally oriented fibrils and, in addition, fibrils within the pore canals that follow their helical shape traversing the cuticle in the vertical direction (Huber *et al* 2014). Here, no particular orientation preference of the calcite grains *c*-axes was observed. In *T. europaeus*, however, the

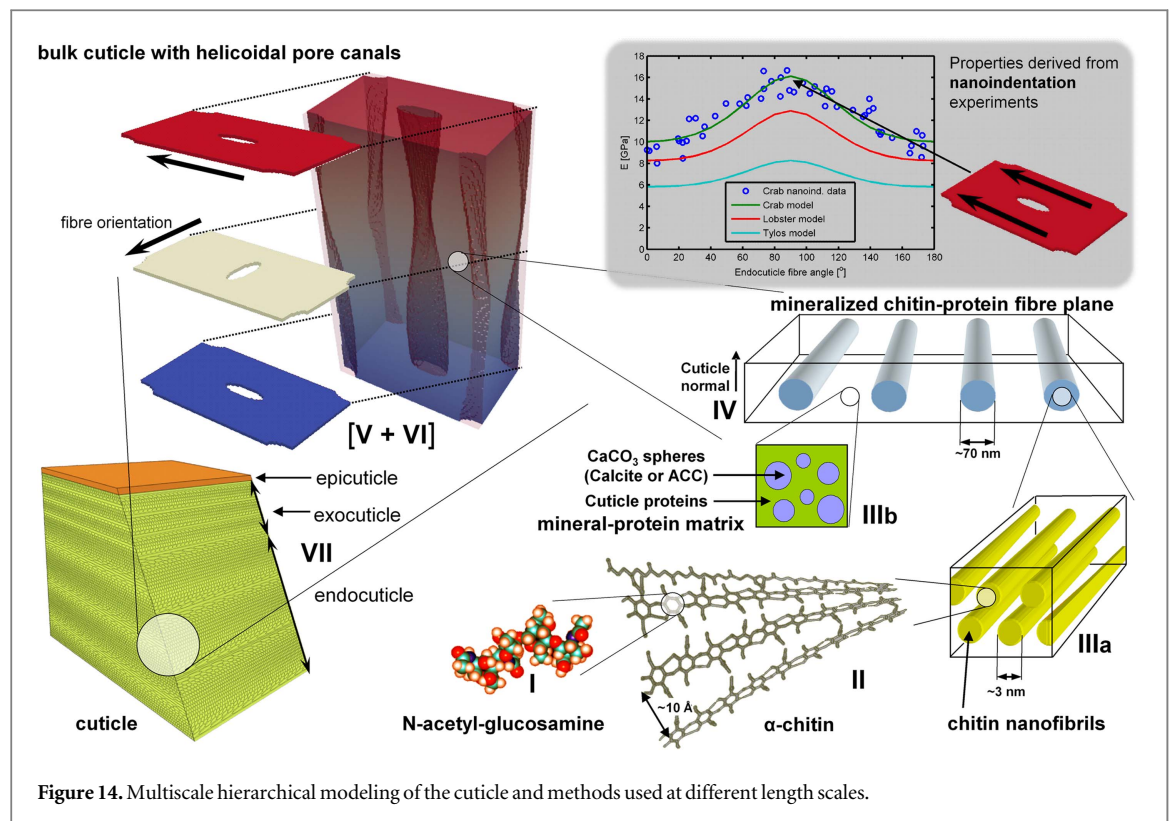
calcite *c*-axis has a strict orientation preference parallel to the surface of the cuticle (Huber *et al* 2015). This can be explained by the lack of pore canals and thus lack of vertically oriented fibers in the distal exocuticle (figure 12). In cuticle without vertical fibrils, orientation of calcite crystals with the cleavage plane in parallel to the fibril orientation would be disadvantageous, because it could facilitate delamination of cuticle layers. In contrast, a *c*-axis orientation preference horizontally (or vertically) maximizes the number of horizontal fibrils piercing through the calcite cleavage planes, making the cuticle more stable. However, such preference in calcite orientation was not observed in the tergites *T. europaeus* (see above) that lack vertically oriented fibers as well. This suggests that a calcite orientation preference as observed in the PI of the species is just required when the cuticle has to sustain high mechanical loads (Huber *et al* 2015).

The most pronounced difference in the morphology of the mandibles of the carnivorous species *C. pagurus* and the omnivore *H. americanus* is the presence of a well-developed PM in the lobster, which is almost completely reduced in the crab (figure 13). The tip of the pars incisiva of mandibles from the decapod *H. americanus* appears to consist of very thick epicuticle only (figure 13(a), table 1). Fractured samples revealed a very high abundance of epicuticular canaliculae oriented towards the tip of the PI within this layer. Structure and composition of the exo- and



endocuticle forming the corpus of the mandible largely corresponds to that of the body shell, except for the central region of the PI that contains ACP, which is replaced by ACC towards the margins and the base. The elastic modulus is 3.7 GPa in the tip and 10 GPa at the base. At the interface with the exocuticle, the epicuticle forms extensions that interlock with mineralized exocuticular fibrils that are oriented parallel to the loading direction of the mandible during feeding. This delicate interface is likely preventing delamination of the two layers (figure 13(b)). In contrast, the surface of the PM (figure 13(c)) is fully mineralized with large amounts of calcite, leading to an elastic

modulus of 30.4 GPa. Towards the base, the mineral phase changes towards ACC and ACP with a resulting lower elastic modulus of 14.1 GPa. The PI of *C. pagurus* (figure 13(d)) consists entirely of twisted plywood mineralized by a calcite-ACC mixture close to the cutting edge, and calcite only in the other regions. This results in elastic moduli of 32.6 GPa and 28.2 GPa, respectively. The epicuticle is not modified in comparison to cuticle forming the body shell. Within endo- and exocuticle, the stacking height of the plywood layers decreases continuously from the base (figure 13(e)) to the tip (figure 13(f)) of the PI. Morphology and fine structure of the mandibles



**Figure 14.** Multiscale hierarchical modeling of the cuticle and methods used at different length scales.

correlate well to the feeding mode of the decapods (Bentov *et al* 2016). The omnivorous *H. americanus* uses a specialized PM for chewing and the PI for grasping food items only, hence a sharp hard edge is probably not essential here. For the carnivorous species *C. pagurus* the Pi is its only cutting tool, which might be the reason for this specific structural organization.

#### 4. Multi-scale modeling

In order to determine the material properties of the cuticle as a function of different exoskeleton compositions, constituents and structural design features, a multi-methodological modeling analysis was performed as a synergic complement to the above discussed experimental research. A very powerful approach, that allows obtaining a much deeper insight and a thorough understanding of the structure-property relations in different materials, is multi-scale modeling (e.g., Buehler 2008, Buehler and Gao 2006). When theoretically describing hierarchical bio-composites, they can be modeled as microscopically heterogeneous bodies containing one dispersed phase (inclusions) embedded in a matrix phase. The objective is then to predict the response of the entire composite body under given loads and boundary conditions based on its microstructure. To solve the problem, a representative volume element (RVE) is defined, which is generally much smaller than the studied body but is large enough to contain the same statistical information about the microstructure as the body itself and responds to given boundary conditions

as a homogeneous piece of matter. Thus the goal of homogenization is to replace the heterogeneous RVE by a homogeneous material with the same averaged properties as the heterogeneous one. Specifically in case of hierarchical bio-composites, their overall complexity is optimally tackled by a complementary combination of quantum-mechanical calculations and higher-scale hierarchical modeling. *Ab initio* methods provide an unprecedented predictive power while their limitations with respect to the size of studied systems are compensated by hierarchical homogenization approaches applied to all relevant higher-level length scales (Buehler and Gao 2006, Nikolov *et al* 2010, 2012, 2015, Fabritius *et al* 2011, Friak *et al* 2013, Friak *et al* 2014, Strelcova *et al* 2016).

A necessary prerequisite for setting up a proper multi-scale model is a detailed structural and compositional description based on experimental findings. These are, however, rather difficult to obtain for the nanoscale and, consequently, many materials properties related to, e.g. proteins, are still unknown. To overcome these difficulties, quantum-mechanical calculations can be applied to determine experimentally inaccessible materials characteristics. The chitin-based mineralized cuticle forming a crustacean exoskeleton, specifically that of the lobster *Homarus americanus*, is below analyzed in detail as an illustrative example. The constitutive model is depicted in figure 14. For each hierarchy level, a RVE of the heterogeneous material is defined and its homogenized properties are found using a suitable modeling method (Mori and Tanaka 1973, Benveniste 1987,

Torquato 1998, Torquato 2002). The properties of each phase forming the composite as well as the volume fractions, the shapes, and the orientations of the dispersed phases are considered as input parameters (Torquato 1998, Torquato 2002). The homogenized properties at the lower hierarchy level are transferred upscale and used in the modeling of the microstructure at the next higher level. Focusing on elastic properties, materials parameters, that are up-transferred in a ‘handshaking’ manner, are the elastic constants. The overall elastic properties are then obtained by repetitive step-by-step application of homogenization methods in a bottom-up order to cover all observed microstructures at all length scales from  $10^{-9}$  to  $10^{-3}$  m. It is noted that the homogenization methods give information only about the average distributions of the stress and strain fields in each phase of the composite. In a more advanced approach (Nikolov *et al* 2015), the local stresses and strains at each material point of the highest-level, macroscopic RVE of the bio-composite are evaluated using a full-field spectral method based on fast Fourier transforms (FFTs) as an alternative to the classical finite element analysis. Further details on this approach are given below.

The lowest relevant level in our multi-scale analysis is the nanometer scale, where the electronic structure and inter-atomic bonding of studied material determine the elastic properties (figure 14(I)). An example material is  $\alpha$ -chitin. Because of the extremely small length scale and the difficulty to obtain and isolate a pure  $\alpha$ -chitin sample, it is not possible to experimentally measure components of the elastic tensor of the chitin nanofibrils (figure 14(IIIa)). Therefore, *ab initio* calculations of single-crystalline  $\alpha$ -chitin were used to get the missing elastic parameters. After identifying the  $\alpha$ -chitin ground-state structure (the lowest energy atomic configuration) including the positions of the H atoms and the H-binding networks, the  $\alpha$ -chitin elastic tensor was determined (Nikolov *et al* 2010, Petrov *et al* 2013). Its analysis showed that crystalline  $\alpha$ -chitin exhibits at the atomistic scale strongly anisotropic elastic properties reflecting the intrinsic nature of the interatomic interactions along the different directions (stiffer covalent bonds along the backbone molecular structure versus softer hydrogen bonds interlinking the molecular chains). The determined elastic parameters were then transferred up-scale to the next scale taking into account experimental information about the actual composite hierarchy reflected in the used multiscale structural model (figure 14).

Next to organic components, like the above discussed  $\alpha$ -chitin or proteins, other building blocks, that are critically important for arthropod exoskeletons, are inorganic ones, e.g. calcite particles (see figure 14(IIIb)). In their case also local chemical variations and inhomogeneity should be taken into account as it was experimentally observed that their

composition may spatially vary (e.g. Boßelmann *et al* 2007, Fabritius *et al* 2009, Seidl *et al* 2014, Huber *et al* 2014). As a consequence of these variations, the elastic parameters (full elastic tensor) correspondingly exhibit local spatial variations, too, following chemical trends. Focusing on the frequently occurring Mg additions in biogenic calcite crystals, parameter-free quantum-mechanical calculations were used to derive chemical trends for thermodynamic, structural and elastic properties of Mg-containing calcite single-crystals (Elstnerova *et al* 2010, Zhu *et al* 2013, Friak *et al* 2014). These studies revealed that the Mg additions in the thermodynamically most stable configurations increase most of the elastic constants of Mg-containing calcite crystals. This relation spans several orders of magnitude in the length scales and links the local atomic chemical variations to the predicted changes of the macroscopic elastic moduli. Earlier speculations that Mg additions in calcite-containing biocomposites occur because of their stiffening impact on the mechanical properties (Becker *et al* 2005) were thus confirmed and a direct insight into the principles underlying this biologically important hardening mechanism was obtained.

Having atomic-scale materials parameters available, a multi-scale approach can be applied to analyze how the mechanical properties change at various hierarchical levels when going from the atomic to the macroscopic scale. A surprising outcome of our study (Nikolov *et al* 2010) is the finding that the overall cuticle anisotropy monotonously decreases when going from nano-scale (highly anisotropic single crystalline chitin) to macroscale (nearly isotropic twisted plywood structure with/out pore canals). As another aspect, the theoretical calculations also provided an insight about how nature balances mutually contradicting functional requirements. Specifically in case of pore canals (see figure 14(B)), they are vital for the lobster because of their role in biomineralization while at the same time they would potentially weaken the structure. The formation of regular honeycombs with thick walls guarantees the largest possible in-plane stiffness compared with other planar microstructures with the same porosity fraction (Nikolov *et al* 2010). This specific geometry ensures that the canal pore area fraction is just as large as to simultaneously ensure optimal cuticle stiffness and maximum weight reduction, thereby efficiently lowering energy costs for the animal when mineralizing the exoskeleton.

Another important aspect that could be analyzed is the structural tolerance of mineralized load-bearing parts of the chitin-based exoskeleton. The robustness of the cuticle behavior with respect to variations (or equivalently error propagation) can originate from a variety of different sources: hierarchy level, properties of the constituents of the specific structure at this level, geometric design parameters such as volume fractions, and the structure itself. Importantly, the lower levels of the structural hierarchy reflect rather general

designing concepts that appear to be valid for all arthropods. In contrast, on the higher levels, significant differences can be found both in the structural organization and in the chemical composition between different taxa, even in closely related species and between distinct skeletal elements (Neues *et al* 2007, Hild *et al* 2008, Fabritius *et al* 2012, Ruangchai *et al* 2013, Alagboso *et al* 2014, Huber *et al* 2015). Regarding the key structures appearing at lower hierarchy levels, the macroscopic behavior of the cuticle was found to be very robust with respect to large variations in the properties of chitin-protein fibers owing to the specific hierarchical structure and the huge contrast in the properties of chitin and fibril proteins (Nikolov *et al* 2011).

Further, more advanced mathematical tools (such as generalization of variational calculus) can be applied to evaluate the efficiency of the overall design (Hyun and Torquato 2000, Torquato 2002). As already indicated by experimental data related to possible variations in the cuticle ingredients and volume fractions, the design was confirmed to be highly optimized and the structural variations were found to be efficiently used to allow for the best possible performance for a given composition (Nikolov *et al* 2010). This can be attributed to the smart hierarchical organization of the cuticle design that leads to an optimal use of the stiffness of the individual ingredients. Again, given the general validity of the used mathematical modeling tools, the approach can be applied to other hierarchical biomaterials without limitations as long as the proper homogenization models are used for each hierarchy level beyond the atomistic scale. A purely continuum version of this approach has been developed to study the elasticity of mineralized collagen fibrils array and the role of extrafibrillar mineralization in bone (Nikolov and Raabe 2008).

Lastly, at the macroscopic level (see figure 14 parts (V + VI)), using detailed experimental characterization and statistics information related the macro-level microstructure, a realistic 3D model geometry may be set up and then analyzed employing a multi-scale FFT spectral method. To each material point of the macroscopic RVE are assigned mechanical properties obtained by hierarchical homogenization at the lower levels. The model may then very closely mimic a specific macroscopic biocomposite, e.g. a specific cuticle of a chosen species. As a major output, the FFT spectral methods can determine, for instance, local distributions of stresses and strains provided that the homogenized data for lower hierarchical levels are known. As an example, the endocuticle of *Cancer pagurus* was studied and it was found that there is an unfavourable orientation of the ellipsoidal cross-section of the pore canals with respect to the loading direction, namely when the long axis of the ellipse is perpendicular to the tensile direction. Stress concentration is then situated at the corners of the ellipsoidal pore canal cross-section perpendicular to the

loading direction. That is very important as the sites of stress concentration are likely also the sites where micro-cracks would initiate. Interestingly, *C. pagurus* reinforces the corresponding sites in the endocuticular pore canals with mineralized fibers (Nikolov *et al* 2015). When the long axis of the ellipsoidal pores and the fibers are oriented along the tensile direction, the average stress is higher but much more uniform and there are almost no stress concentration sites. In addition, the cuticle has a higher elastic modulus in this direction because of the chitin fibers. Thus, the brittle endocuticle seems to possess an effective crack arrest mechanism that allows for more damage before failure.

## 5. Conclusions

Given the enormous diversity of different species and the corresponding evolutionary adaptations of the Crustacea, our review is far from providing a complete picture of the real diversity present in the structural and compositional organization of their exoskeletal elements. What can be said is that in order to achieve specific properties required for the optimal function of a skeletal element, the crustacean cuticle can be modified in every aspect of its organization above the level of the chitin nanofibrils, which are the essential organic building blocks. Given the fact that little is known about the local composition of the proteins covering the chitin, which may also vary among species and within different skeletal elements, even this might not be the final limitation. For the organic phase of the cuticle, we observed a high variability of the structural organization within the higher levels of the structural hierarchy above the fibril/fiber layer level (figure 1, level (V)). We observed all kinds of modifications in the helical pitch of twisted plywood including unidirectionally oriented fiber layers that can occur in exo- and endocuticle. These layers can vary in thickness by the varying number and density of fibers. We show that the epicuticle, whose function is frequently believed to be reduced to acting as a chemical barrier to the environment, can actually be highly important for the function of certain skeletal elements. Even the number of hierarchical levels a specific cuticle type can comprise is not fixed. Specifically, the formation of large chitin-protein fibers (figure 1, level (IV)) does not occur in all studied examples and must be considered much more widespread within Crustacea than expected. Except for some special cases such as arthroal membranes, the exo- and endocuticle are always mineralized. Our examples show that the animals are able to control the mineral phase, morphology and crystallographic orientation. These parameters can even vary within each layer, e.g. both exo- and endocuticle can contain several different mineral phases in different regions but also co-localized. This adds additional degrees of

freedom to tailor the properties of skeletal elements. Even more variability is provided by the variable geometry and morphology of the pore canal system. The animals are capable of controlling these factors to e.g. influence the mechanical properties or to create interfaces that increase the toughness of skeletal elements.

As a synergic complement to experimental research, multi-methodological scale-bridging modeling approaches represent an optimum strategy for analyzing hierarchical biocomposites. The modeling methods (such as in Buehler and Gao 2006, Nikolov et al 2010, 2011, 2012, 2015, Fabritius et al 2011, Friak et al 2013, 2014 or Strelcova et al 2016) allow examining local structural and chemical variations as well as the overall robustness of biocomposites design. The fact that different types of variations are included is crucial as these variations often occur in nature, e.g. those caused by different stages of growth, molt cycle, injuries, and/or synthesis heterogeneity. All these aspects result in the properties and the volume fractions of the tissue constituents that locally may considerably vary. Experimentally validated physics-based scale-bridging models are an excellent vehicle to conduct structure-property tolerance studies as the different ingredients and their behavior can be systematically varied.

A thorough analysis of the structure-properties relations of some constituents in biological hard tissues (e.g., proteins) is crucial also for possible biomimetic applications. It should be noted that the aim is not to reproduce certain properties of biological matter in a synthetic material by simple copying the original biological structure but to rather understand the mechanical principle behind the material and replace the underlying building blocks or their topological connectivity. Hierarchical modeling approaches may identify the most important ingredients that are decisive for a specific property as well as the tolerance of the overall material behavior against local structural or chemical variations. Conclusions resulting from synergic studies combining experiments and modeling suggest that real biological materials often reveal an optimal use of their ingredients regarding the best possible performance for a given composition.

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