

The Cuticle of Krill (*Euphausia superba*) in Comparison to That of Other Crustaceans

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Periodical reconstruction of the exoskeleton during the moulting cycle is certainly one of the most energy consuming processes crustaceans have to go through. In an attempt to understand the main factors that influence the growth of krill, special attention is directed to their cuticle. A detailed comparative study of the morphology and histochemistry of the krill cuticle is one step to evaluate adaptations of euphausiids to their environmental conditions.

Depending on body location, animal size and species, the crustacean cuticle varies considerably in thickness and degree of mineralization. Common features in the composition of crustacean cuticles are chitin/protein microfibrils, arranged in thin lamellae at the epidermal surface, parallel to their long axis. In every subsequent lamella the main direction of the microfibrils is rotated by a certain angle around an axis perpendicular to the lamellae. When an overall rotation of 180° is reached, the matrix of a cuticular lamina is complete (Bouligand, 1965, 1972; Neville, 1975; Giraud-Guille, 1984). Tanning and mineralization confer rigidity to the cuticle.

Morphologically there are at least three different layers obvious in the crustacean cuticle: An outermost epicuticle is followed by an exocuticle (synonyms: couche pigmentaire; procuticle 1) and an endocuticle (couche principale; procuticle 2) (Schultz & Kennedy,

1977; Foster & Howse, 1978; Arsenault *et al.*, 1984). The new epi- and exocuticle are already deposited before a crustacean sheds its old shell in the moult. The endocuticle is quickly built up in the postmoult period. In most of the investigated decapod and copepod crustacea an additional membranous layer (couche membraneuse) is found between the endocuticle and the epidermis. It is the last layer deposited and is never mineralized (Drach, 1939; Skinner, 1962; Travis, 1965; Bouligand, 1971; Green & Neff, 1972; Gharagozlou van Ginneken & Bouligand, 1973, 1975).

This paper deals with the general intermoult morphology, whereas the dynamic aspect of cuticle production in relation to the moult cycle will be published elsewhere in more detail.

MATERIALS AND METHODS

Juvenile *Euphausia superba*, 27 to 37 mm long, were caught in Admiralty Bay, King George Island, South Shetland Islands and maintained in aquaria at the Polish station "H. Arctowski". Some specimens taken fresh from the field were also studied, serving as controls to rule out possible morphological changes caused by the specific maintenance conditions. After determination of moulting stages (Buchholz, 1982) the animals were sacrificed and their most distal pleon segments detached with new razor blades. The sixth pleon

segments were quickly immersed in 6.25% glutaraldehyde in 0.15M phosphate buffer for light and electron microscopy. The fifth pleon segments were covered with Bouin's fixative or 4% formalin + 0.5% cetylpyridine for light microscopy.

After three weeks to four months in the fixatives at 4°C, the samples for electron microscopy were repeatedly rinsed in cold 0.1M phosphate buffer with 7.5% sucrose (pH 7.6-7.8), postfixed for 6 hours in 2% phosphate-buffered OsO₄, dehydrated in a graded series of ethanol, passed through propylene oxide and embedded in araldite. Thin sections were mounted on formvar coated grids, double stained with a saturated solution of uranyl acetate in 70% methanol and lead citrate according to Reynolds (1963) and viewed in a Siemens Elmiskop 101.

Semithin sections were used for histochemistry after removal of the araldite by a saturated solution of sodium hydroxide in absolute ethyl alcohol (Lane and Europa, 1965). In addition, OsO₄ was carefully removed by 2% H₂O₂ for the alcian blue reaction.

Samples fixed in Bouin's were conventionally processed and embedded in paraplast. Sections 5-10 µm thick were used for histochemical study.

To avoid loss of sections during histochemical procedures adhesion of araldite and paraplast sections to slides was enhanced by keeping them at a temperature of 65°C for at least one night.

Histochemistry

The PAS reaction for glycoproteins and glycogen was conducted after McManus (in Pearse, 1985). Nuclei were counterstained with Weigert's hematoxylin.

The alcian blue test, pH 2.5, for carboxyl-rich glycoconjugates was performed after Steedman (1950, in Pearse, 1985) on semithin sections with or without OsO₄. The Baker Modification of the Millon Reaction was sought to detect tyrosine (in Pearse, 1985).

RESULTS

Electron micrographs of intermoult krill cuticle show the fully developed exoskeleton consisting of three layers (Fig. 1). In

transverse sections of sixth pleon segments cuticle thickness varies between extremes of 3.2 µm in a ventral area and 15.5 µm dorsally. The mean value for lateral and dorsal parts is 9.7 µm ± 3.7 (n=10).

Epicuticle

The krill epicuticle occupies approximately 1.6% of an average cuticle diameter. It is approximately 160 nm thick with a large variation, due to conditions of preservation of the surface coat. It is easily detached from the underlying exocuticle and the resulting artificial gap is often filled with electron dense material, frequently crystalline and of unknown nature. The epicuticle contains four sublayers (Figs. 2 a-c): An electron dense surface coat (1) is in contact with the surrounding sea water. It is followed by an electron translucent (2) and an electron dense continuous layer (3). The innermost layer of the epicuticle (4) shows closely spaced round to oval electron dense structures. An oblique section of the sublayers (Fig. 2c) demonstrates that the round structures in sublayer 4 are not transverse sections of parallel rods, but rather spheroidal structures. Sublayer 4 frequently resembles a simple line, because the sections are usually thicker than the distance between two rows of the spheroids. The spheroids deeper in the section optically fill the gaps between the upper ones. At irregular intervals indentations (Fig. 2a) interrupt the prevailing structure of the epicuticle. In these areas sublayer 4 is missing and instead of the surface coat, electron dense "clots" (asterisk in Fig. 2a) of unknown chemical nature are inserted.

The epicuticle is the only PAS-positive cuticular layer, indicating the presence of glycoproteins. The reaction is more easily shown on the thicker paraplast sections than on the equally positive 1 µm araldite sections, because an oblique section through 5 µm section thickness artificially dilates the epicuticle (Fig. 3).

In the carboxyl-rich glycoconjugates and tyrosine tests the epicuticle is either not stained or cannot be distinguished from the underlying exocuticle.

Exocuticle

The epicuticle, as well as the new exocuticle of *E. superba*, is deposited in the

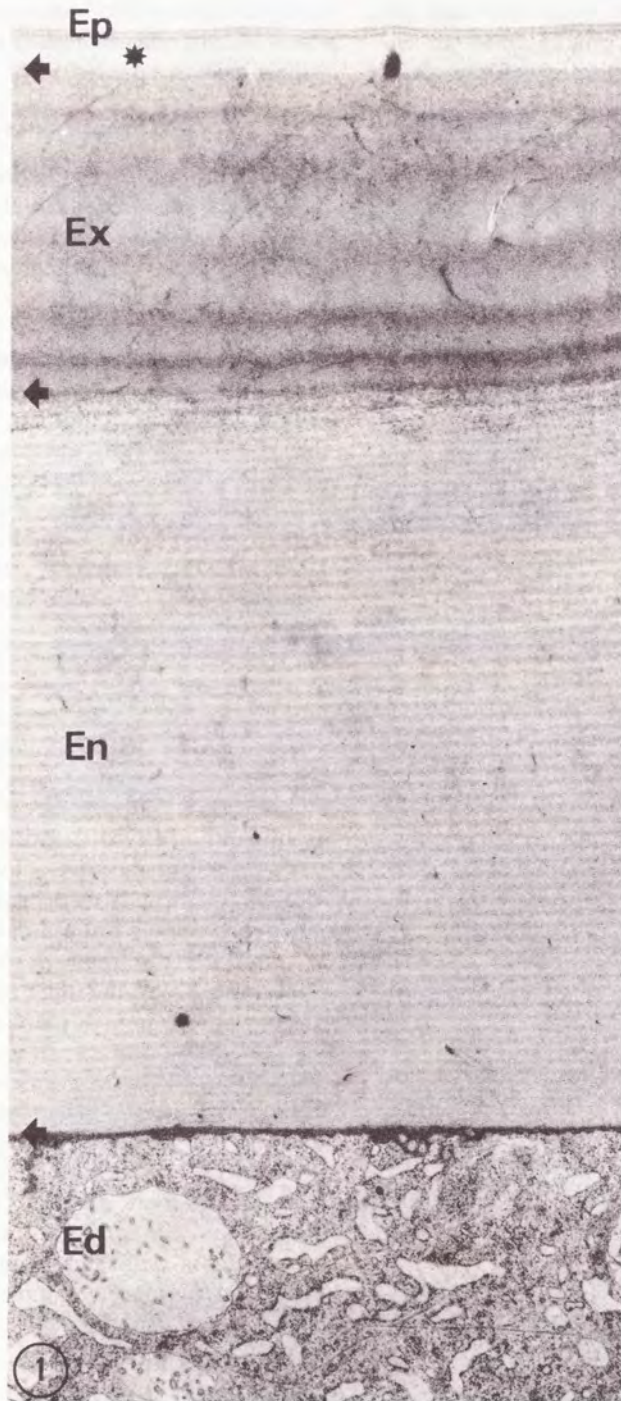


Fig. 1 – Fully developed cuticle of *E. superba* in moulting stage D₀. The exuvial cleft between epidermis and cuticle has just started to open. Arrows indicate extensions of cuticular layers. The asterisk marks an artificial gap usually found between epi- and exocuticle. Ed, epidermis; En, endocuticle; Ep, epicuticle; Ex, exocuticle. x 12000.

premoult period. It has a laminated structure with laminae of different thicknesses, 80% of the laminae measuring between 300 and 600 nm.

The exocuticle is stained by the alcian blue method, pH 2.5, for carboxyl-rich glycoconjugates and stains yellowish red by the Millon Reaction, revealing the hydroxy-phenyl group of tyrosine in this part of the cuticle (Figs. 4 and 5). In premoult stages the newly produced exocuticle beneath the old cuticle has the same staining properties as the old exocuticle.

Endocuticle

The krill endocuticle can become the thickest cuticular layer as is common in other crustaceans, but it is not obligatory in *Euphausia*. Frequently exo- and endocuticle are approximately the same size. The endocuticle of krill in contrast to decapod endocuticle shows much thinner laminae than the exocuticle (Fig. 6). Eighty percent of the endocuticular laminae are 120 to 160 nm thick. They are laid down from early postmoult (moulting stage A-) to late stage BC (Buchholz, 1982). Laminae thickness is reduced as the postmoult period progresses.

In the endocuticle there are neither carboxyl-rich glycoconjugates nor tyrosine detectable by histochemistry.

Pore Canals

Pore canals originally containing epidermal cell processes are obvious beneath the epicuticle in the exocuticle (Fig. 2c) and the oldest laminae of the endocuticle. In the proximal endocuticle there are hardly traces of pore canals. With the growing cuticle these pore canals seem to become narrower and are at last cut off as the endocuticle is completed. It is not known when the epidermal processes are withdrawn or what their fate is.

Tonofibrils

In addition to pore canals tonofibrils

transgress endo- and exocuticle in great numbers in the muscle attachment areas. Tonofibrils serve to fasten muscle fibers in the exoskeleton via intermediate tendinal cells (Rossner & Sherman, 1976).

DISCUSSION

It is well established that the fully developed crustacean cuticle consists of four layers (epi-, exo-, endocuticle and the membranous layer), whereas in krill we found only three layers.

The pleon epicuticle is 10 to 25 times thinner than that of crabs and crayfish (Kümmel *et al.*, 1970; Green & Neff, 1972; Giraud-Guille & Quintana, 1982), but about the size of the gill epicuticle of the shrimp *Penaeus* (Foster & Howse, 1978). Epicuticles of some copepods and cladocerans (Gharagozlu van Ginneken & Bouligand, 1973, 1975; Halcrow, 1976; Schultz & Kennedy, 1977) measure only a tenth to a fifth of the krill epicuticle.

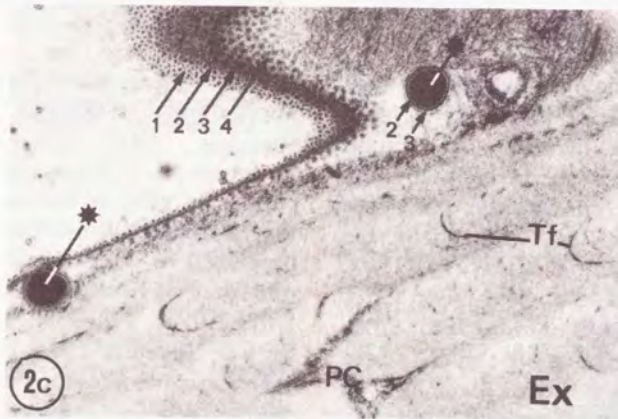
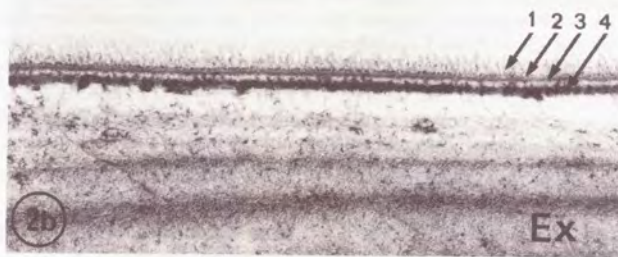
Epicuticular thickness is not only related to differing body size and total cuticle thickness, but also based on structural differences as is particularly striking on the ventral and dorsal cuticle of the copepod *Porcellidium* (Gharagozlu van Ginneken & Bouligand, 1975). Body epicuticles of decapod crustaceans intrude into the exocuticle by a fifth or sixth, most extensive sublayer called "protein-epicuticle" (Kümmel *et al.*, 1970; Green & Neff, 1972). This "anchorage" of the epi- in the exocuticle is usually absent in krill pleon segments.

In only one of 70 *E. superba* and 30 *Meganocyttiphanes norvegica* pleon segments, branchiostegites and uropods (unpublished results), we found a faint resemblance to an "anchoring" looking very much like the epicuticle Albert & Kils (1983) saw on gill cuticle of *E. superba*. Consequently, the epicuticle is detached from the exocuticle in the majority of our samples, resulting in an artificial gap or loss of the outermost layer.

Fig. 2 - Epicuticle of *E. superba* with sublayers 1 to 4. Asterisks mark electron dense clots in paramedial side view (a) and in more lateral side and top view (c) interrupting the prevailing epicuticular structure at irregular intervals. Ex, exocuticle. Extremely thin section. Sublayer 4 appears as a row of ball-like structures. $\times 80000$. b) Normal ultrathin section. Sublayer 4 is more easily recognized as a surface coat. Sublayer 4 appears as a simple line. $\times 20000$. c) Transverse to oblique section. Sublayer 4 consists of spheroidal and not rod-like structures as the top view shows. PC pore canal; Tf tonofibrils. $\times 20000$.
Fig. 3 - Krill integument stained with PAS. Epicuticle and basal membrane show a positive, exo- and endocuticle a negative reaction to PAS. Bm, basal membrane; Ed, epidermis; Ep, epicuticle. $\times 640$.

While Travis (1965) described the newly synthesized epicuticle of *Orconectes* as being PAS positive only around the moult, we found the krill epicuticle stained by the PAS reaction in all moulting stages. Moreover, this applied

as well to the old as to the newly synthesized epicuticle and suggests the presence of glycoproteins in both structures. In analogy to the morphologically similar cell surface coats, glycoproteins could be expected in the



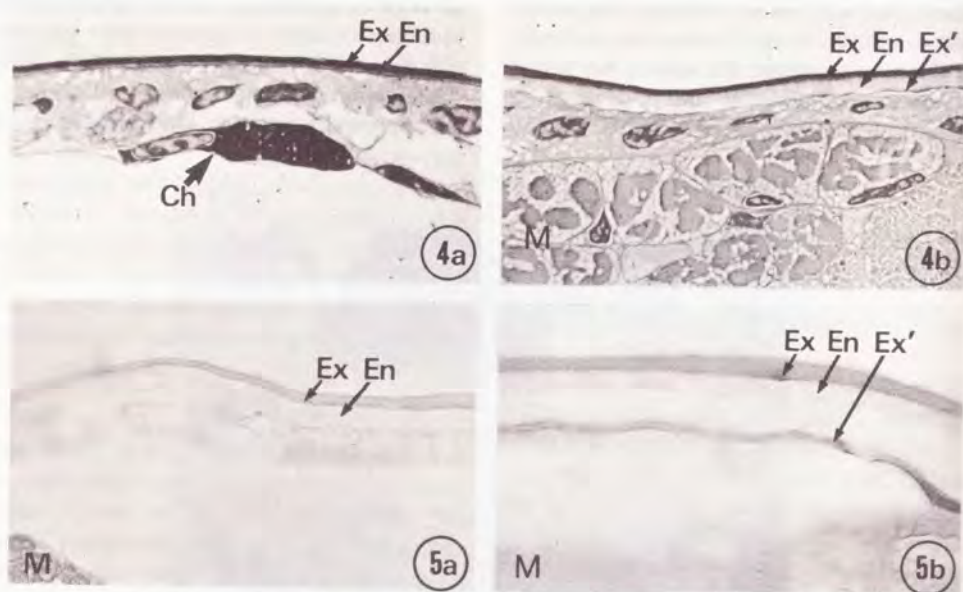


Fig. 4 - Semithin sections of krill integument stained with alcian blue. Only the old and new exocuticles show a positive reaction to alcian blue. OsO_4 was not removed from these sections and is responsible for the background stain. Ch, chromatophore; En, endocuticle; Ex, exocuticle; Ex', new exocuticle; M, muscle. a) Moulting stage BC. The still thin endocuticle is not stained. A chromatophore is intensely impregnated by OsO_4 . $\times 400$. b) Moulting stage D_1 . $\times 400$.

Fig. 5 - Paraffin sections of krill integument after Millon-Reaction. Tyrosine is stained in the exocuticles and in muscle tissue. The endocuticle is always free of stain. a) Moulting stage BC. $\times 400$. b) Moulting stage D_2 . $\times 400$.

epicuticular surface coat. In the case of krill, glycoproteins on its outer surface can reduce water friction (Bond, 1979) and thus lower the energy cost of their continuous swimming.

In krill the epicuticle covers a layer with relatively thick laminae followed by a layer with distinctly thinner laminae. In decapod crustacea the sequence of the layers below the epicuticle is reversed (Fig. 6b), the exocuticle showing the thinner, the endocuticle the thicker laminae (Skinner, 1962; Travis, 1965; Green & Neff, 1972; Foster & Howse, 1978; Demeusy, 1980; Arsenault *et al.*, 1984). Since in euphausiids the membranous layer, where present, is built of much thinner laminae than the endocuticle, the morphological study had to be supplemented by histochemical tests in order to determine whether the exocuticle or the membranous layer was reduced.

There were several indications suggesting the hypothesis that it was not the exocuticle, but the membranous layer that was missing:

A first hint was that the innermost layer of krill cuticle can be its thickest part, while the studied membranous layers are rather thin

and the endocuticle is generally reported to be the thickest layer.

Secondly, in some publications on cuticle structure the membranous layer is neither mentioned nor shown in the micrographs (Halcrow, 1976; Schultz & Kennedy, 1977; Arsenault *et al.*, 1984). Furthermore, it is not present in the quite thin cuticle of gills as far as can be judged from the micrographs, most of them predominantly published to demonstrate the very specialized gill tissue (Foster & Howse, 1978; Alberti & Kils, 1983; Finol & Croghan, 1983, and further literature cited there). It seems, as if building a membranous layer is not completely compulsory in crustaceans and it might well be missing in krill.

The literature analysis about entomostracan cuticles revealed that in the thin, light-weight cuticles of copepods and cladocerans, laminae thickness was either more or less the same across all of the cuticle (Gharagzlou van Ginneken & Bouligand, 1973; Halcrow, 1976; Schultz & Kennedy, 1977) or the exocuticle (procuticle 1) had the thicker laminae as is the case for krill

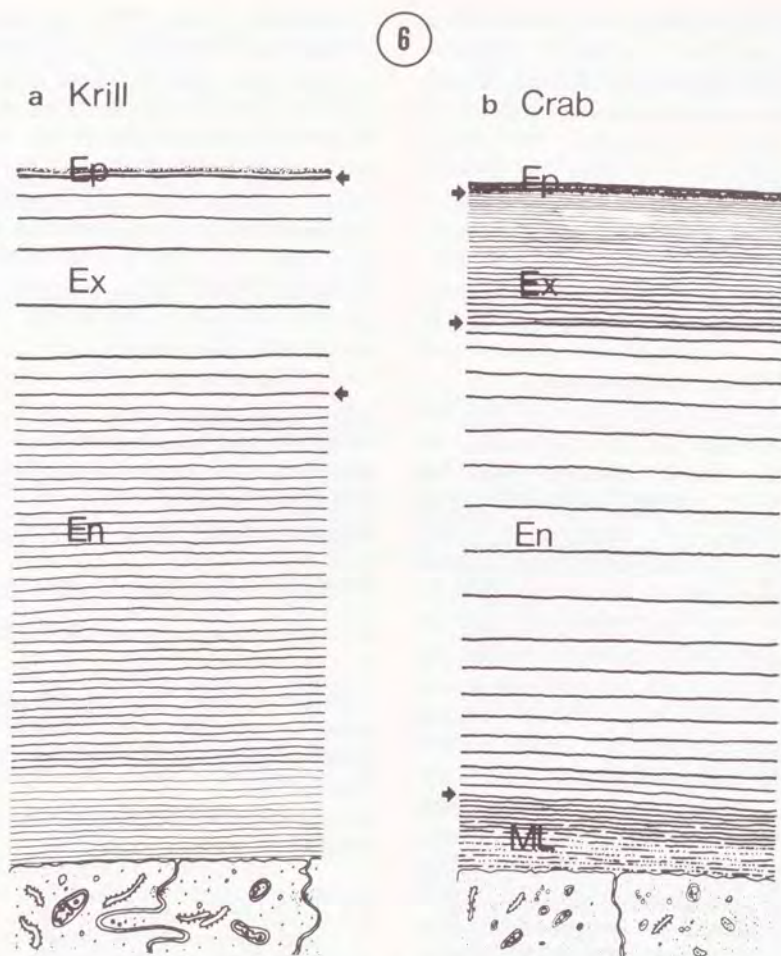


Fig. 6 - Schematic drawing of krill and crab cuticle comparing the number and relative thickness of layers and relative thickness of laminae in the various layers. Ep, epicuticle; Ex, exocuticle; En, endocuticle; ML, membranous layer; arrows mark limits of layers.

(Bouligand, 1962; Gharagzlou van Ginneken & Bouligand, 1975). Particularly interesting in this context is the branchiostegite cuticle structure of the pelagic larvae of *Penaeus aztecus*, where exo- and endocuticle look much as they do in krill (Talbot *et al.*, 1972), while gill cuticle of the benthic subadult animals shows "closely spaced" laminae in the exo- and "broadly spaced" laminae in the endocuticle. The relative thickness of cuticular laminae does not obviously define the cuticular layer.

Another question to consider is in what period of the moulting cycle are the layers synthesized. In crustaceans, epi- and exocuticles are synthesized in the premoult period, while all or most of the endocuticle

and the membranous layer are constructed after the moult (Drach, 1939; Skinner, 1962; Green & Neff, 1972). The krill epi- and "exocuticle" (Fig. 6a) are likewise ready in the last moulting stage before ecdysis. After the moult there is only one more cuticular layer deposited (Buchholz & Buchholz, in preparation). This again corroborates the hypothesis.

The final argument for the existence of the krill exocuticle and the lack of a membranous layer is given by our histochemical results. They show the amino acid tyrosine and carboxyl-rich glycoconjugates exclusively in the exocuticle and the krill endocuticle free of both. According to Travis' investigations on

Orconectes (Travis, 1965), the membranous layer as well as the exocuticle contains tyrosine. Only the endocuticle is free of any reaction product with tyrosine. Had the krill endocuticle been comparable to other crustaceans' membranous layers, the stained and unstained cuticle parts in Figures 4 and 5 would have had to appear reversed. Carboxyl-rich glycoconjugates were not only demonstrated in the exocuticle of *Orconectes* (Travis, 1965) but also in *Penaeus* (Foster & Howse, 1978), while endocuticle and membranous layer did not show them.

We think it sufficiently proven that the krill cuticle consists of an epicuticle, an exocuticle with thick laminae and an endocuticle with thin laminae. The membranous layer is missing in the euphausiids we investigated. One could interpret the membranous layer as the product of an epidermis that has gradually lost its capacity to produce well-ordered laminae. In other crustaceans the membranous layer is laid down in a phase (C_3), when cuticle construction comes to an end (Drach, 1939). Our experimental krill was caught during the austral summer. Its intermoult period is very short during this season (approx. 14-17 days, see Buchholz, 1985) and the epidermis hardly ever ceases to build up cuticular layers (Buchholz & Buchholz, in preparation). The construction of the fourth layer might simply be skipped.

Moreover, as far as lamina thickness is concerned, the cuticle pattern in krill is different from that of juvenile and adult decapods. It compares best to the integumental structure of copepods, cladocerans and a pelagic decapod larva.

Considering size, the thinnest parts of *Euphausia* pleon cuticle ($3.2 \mu\text{m}$) are comparable to thick parts of entomostracan cuticles (e.g. *Daphnia* carapax $4 \mu\text{m}$).

Even though euphausiids, like decapod crustaceans, belong to the subclass Malacostraca, the fine structural aspect of the krill cuticle appears much more delicate than that of decapod crustaceans and more similar to that of Entomostraca. This could be attributed to different ratios of protein and chitin in the different cuticles. For a first orientation, the protein/chitin ratio in krill cuticle was determined according to Welinder's method (Welinder, 1974) to obtain

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comparable data. The separation of the protein/chitin complex in cuticles is critical and different methods result in a very different yield of chitin (Yanase, 1975). Comparison of Welinder's determinations on crayfish, crabs, and shrimps with krill data ($n=6$) indicate a higher proportion of protein in the euphausiids. The protein/chitin ratio is >1 in krill and <1 in the decapod crustaceans Welinder tested. More detailed investigations on the cuticle composition of krill are in progress (F. Buchholz).

Comparisons of the crustacean cuticle with insect cuticle are plenty. Comprehensive structural as well as biochemical comparisons between the cuticle patterns in the different systematic orders within the class Crustacea are rare and should be undertaken.

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SUMMARY

The intermoult cuticle of euphausiids consists of three layers: the outermost epicuticle, the exocuticle and the endocuticle. A membranous layer, as usually found between endocuticle and epidermis in crustaceans, is lacking. In comparison to other crustaceans as well as to total cuticle thickness, the krill epicuticle is thin. Unlike exo- and endocuticle the epicuticle is PAS positive, suggesting that the surface coat found on the four-layered epicuticle consists of glycoproteins. A glycoprotein-coat can reduce water friction and thus save energy needed for swimming.

In krill as in other crustaceans exo- and endocuticle are built up of stacks of lamellae called laminae. In decapod crustaceans the endocuticle shows the thicker laminae, whereas in krill it is the exocuticle. The missing layer is the membranous layer and not the exocuticle, as was verified by histochemical tests for tyrosine and carboxyl-rich glycoconjugates. The periods of synthesis of the various layers in the moulting cycle support this conclusion.

The krill cuticle shows morphological similarities to the light weight cuticles of some entomostracans and the pelagic larvae of shrimp.

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