

# Notes on the integument of the glacier-dwelling tardigrade *Hypsibius klebelsbergi* MIHELČIČ, 1959 (Tardigrada)\*

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**ABSTRACT.** – The integument and muscle attachments of the heavily pigmented glacier tardigrade *Hypsibius klebelsbergi* MIHELČIČ, 1959 were studied by electron microscopy. The transparent cuticle consists of the outer and inner epicuticle, the trilaminar layer, and the procuticle. The latter may be occasionally differentiated in a more electron dense distal and a less dense proximal portion. Muscle attachments correspond to those known from other Tardigrada. Epidermal cells are characterized by an extraordinary number of pigment organelles (granules) that may be interpreted as protection against harmful UV-radiation. Only a few epidermal cells lack the pigmentation, e.g., those at the muscle attachment sites. These pigment-free areas, some kind of “windows” that may allow penetration of UV-radiation, are markedly reduced by adjacent pigmented epidermal cells which often extend under the pigment-free cell and surround the distal part of the muscle cell.

**KEYWORDS:** Tardigrada, TEM, *Hypsibius klebelsbergi* MIHELČIČ, integument, pigmentation, muscle attachments, UV-protection, glaciers

## Introduction

Tardigrades can be found in a variety of extremely different habitats ranging from bryophyte or lichen cushions in the highest mountains to the sediments of the abyssal bottom (e.g., EHRENBURG 1859, MARCUS 1927, 1929, RENAUD-MORNANT 1983). However, to the most remarkable habitats belong cryoconite holes, i.e., microcaverns on the ice surface in the glaciers' ablation zone. The holes are caused by solar radiation on dark particles on the ice surface (e.g., VON DRYGALSKI 1897, STEINBÖCK 1936, 1957, GRØNGAARD et al. 1999); they are predominantly water-filled and frozen for most of the year. Three tardigrade species, *Hypsibius klebelsbergi* MIHELČIČ, 1959, *H. janetscheki* RAMAZZOTTI, 1968 and *H. thaleri* DASTYCH, 2004 have been exclusively reported from these habitats (MIHELČIČ 1959, RAMAZZOTTI 1968, KRAUS 1977: unpublished PhD thesis, JANETSCHEK 1990) and, according to recent studies (DASTYCH et al. 2003, DASTYCH 2004a, b), they

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\* In memoriam Univ. Prof. Dr. Konrad Thaler (Innsbruck), obiit 11.07.2005.

should be considered as true cryobionts. These extremely cold-stenotherm species are heavily pigmented and dwell not only at the temperature of 0 °C or very close to (often freezing completely with their surrounding), but are also exposed to very high UV-radiation. The latter is additionally amplified by strong albedo, a characteristics of the glacial environment. The markedly dark pigmentation in adults of these species, particularly of *H. klebelsbergi*, has been recently examined in *LM* by DASTYCH et al., DASTYCH (*l.c.*).

Black pigments are widely distributed in the integument of invertebrates and vertebrates exposed to UV-radiation. Therefore, as in the mentioned tardigrades, the heavy pigmentation is suggested as being protective against harmful UV-radiation (for glacier invertebrates see, e.g., GOODMAN & PARRISH 1971, EISENBEIS & MEYER 1986, 1999, DASTYCH et al. 2003).

Colouring of tardigrades may be caused by the intestine contents, carotenoids in the body cavity, the epidermis, the cuticle (e.g., the Echiniscidae) and/or a variety of black and brownish shades occurring mainly in the epidermis (most Eutardigrada). Integumentary colouring may be uniformly distributed, e.g., in the Echiniscidae, *Milnesium tardigradum* DOYÈRE, 1840, some ecomorphs of *Hypsibius dujardini* (DOYÈRE, 1840), or its distribution shows a distinct pattern, e.g., in *Ramazzottius oberhaeuseri* (DOYÈRE, 1840). The ventral side of some tardigrades seems to be less pigmented than their dorsum. Generally, epidermal portions at the sites of the muscle attachments are poor in pigment organelles.

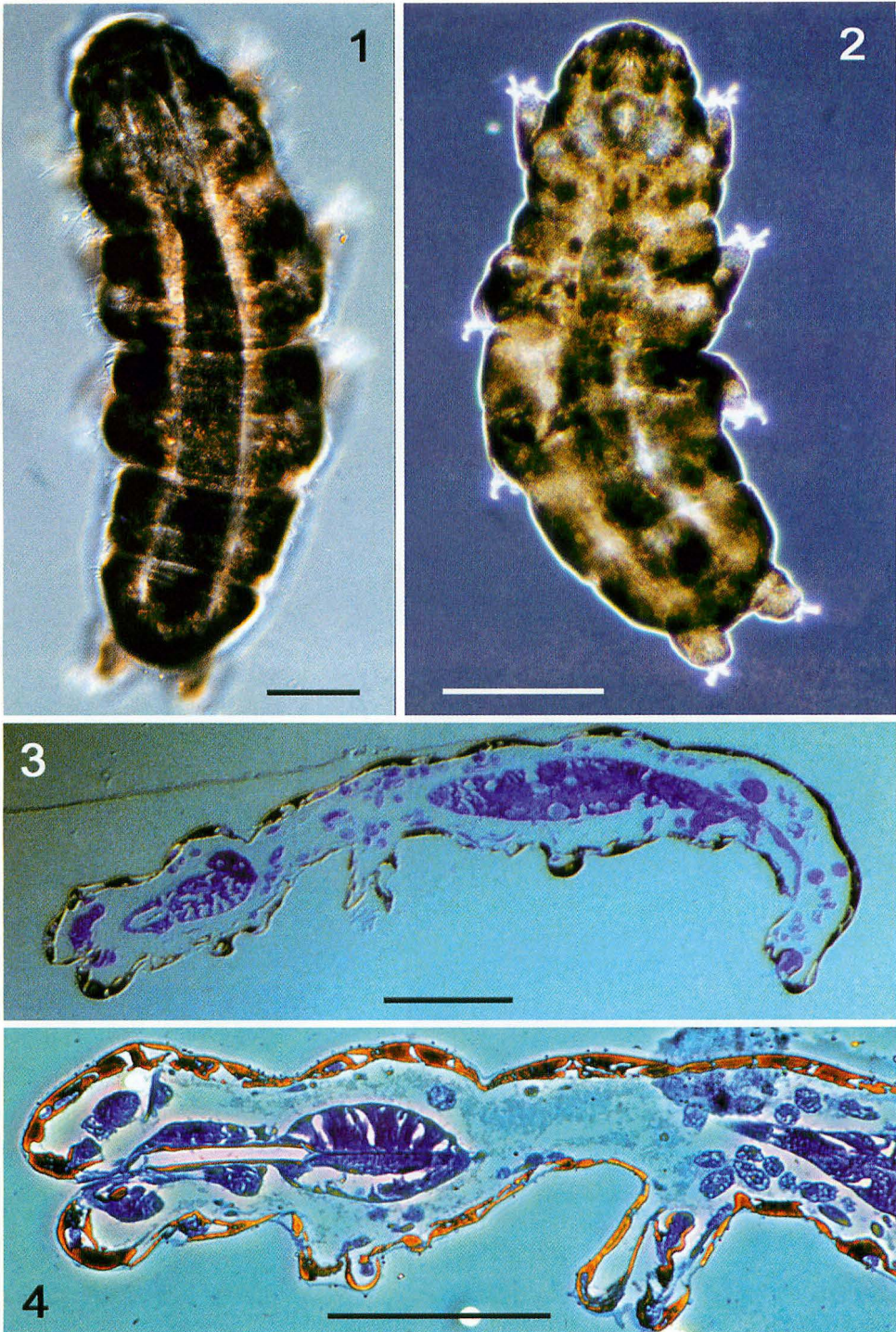
The markedly dark colouring of *H. klebelsbergi*, noted for the first time by STEINBÖCK in the (Stubai) Alps in 1938 and reported under the term “schwärzlichen...Tardigraden” (1957: 69; then still not described species), and data gained at the re-description of this taxon (DASTYCH et al. 2003) prompted us to undertake an ultrastructural study of the integument of this tardigrade. The objective of this paper is to document the general organisation of the cuticle and the epidermis in *H. klebelsbergi*, to show the amount and distribution of the brown-blackish pigment and to look for areas free of pigmentation (“windows”) where UV- radiation may unhindered penetrate the integument.

## Material and methods

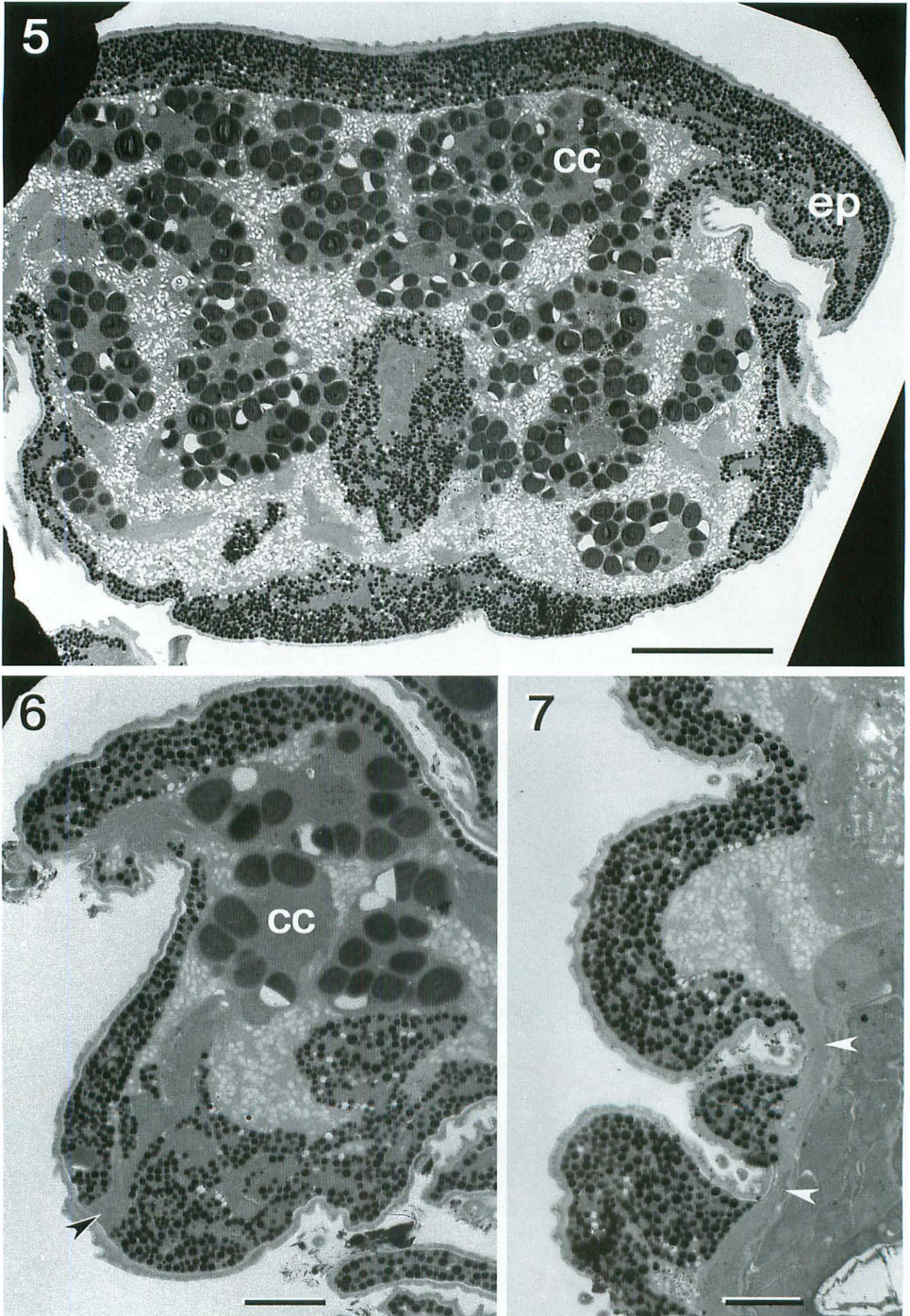
Abbreviations used are: *cc*- cavitory cells, *ep*- epidermis, *DIC*- differential interference contrast, *ie*- inner epicuticle, *LM*- light microscopy, *mu*- muscles, *nu*- nucleus, *PHC*- phase contrast, *pr*- procuticle, *oe*- outer epicuticle, *rer*- rough endoplasmic reticulum, *SEM*- scanning electron microscopy, *TEM*- transmission electron microscopy, *tr*- trilaminar layer.

Specimens of *H. klebelsbergi* were collected from the Rotmoosferner glacier (46°52'N, 11°02'E) near Obergurgl in the Ötztal Alps (Nordtirol, the Austrian Central Alps: 1 September 2004) by the method described in DASTYCH et al. (2003). The material has been brought alive to Hamburg and partly used for observations in *LM*. Fully dark coloured adults ranging from c. 250 to 400 µm in length were water-mounted on microslides and examined with *PHC*- or *DIC* microscope. Semi-thin sections were prepared as described in DASTYCH et al. (*l.c.*). Photomicrographs were taken with ZEISS “Photomikroskop III” and “Axiomat”.

For *TEM* tardigrades were fixed in KARNOVSKY’s fluid (KARNOVSKY 1965) using either 0.1 mol/l phosphate buffer or cacodylate buffer (pH approx. 7.2). Specimens were postfixed in 1% osmiumtetroxide in the corresponding buffer and embedded in SPURR’S medium (SPURR 1969) or fixed either in 2.5 or 5 % glutaraldehyde and embedded in Epon or Durcupan ACM, respectively. Ultrathin sections were cut with diamond knives, stained with lead citrate (REYNOLDS 1963) and viewed in a ZEISS EM 9-S2 or in an EM 902 electron microscope.



**Figs 1-4.** *Hypsibius klebelsbergi* MIHELČIĆ: **1**, dorsal- ; **2**, sub-ventral view of two different, water-mounted specimens; **3**, view of the pigmentation in the longitudinal (semi-thin) section of the body (the sub-median vertical plane); **4**, as above, anterior part of the body (median vertical plane) (Figs 1, 3: *DIC*, Figs 2, 4: *PHC*. Bars – Figs 1-4: 50  $\mu$ m).



**Figs 5-7.** TEM of the integument of *Hypsibius klebelsbergi* MIHELČIĆ: low power micrographs, parasagittal; **5**, note the different pigmentation of dorsal (on the top) and ventral (below) epidermis (*ep*, cavitory cells = “hemocytes”(cc) and the putative muscle attachments site in the middle; **6**, part of the ventral side; **7**, part of the dorsal side; note muscle attachments (arrowheads) (Bars – Figs 5-7: 5  $\mu$ m).

## Results

As reported in a previous paper (DASTYCH et al., 2003), most individuals were totally dark, their shades ranging from deep brown to black. Some areas at muscle attachments or along two main muscle cords appeared to be somewhat paler (Figs 1, 2).

Semi-thin sections and low power TEM-pictures showed the integument consisting of a thin cuticle that covers the body and a monolayered epidermis with a mass of pigment granules. In the ventral epidermis, the granules appear to be less abundant and at the area of muscle attachments they seem to be absent (Figs 3-7).

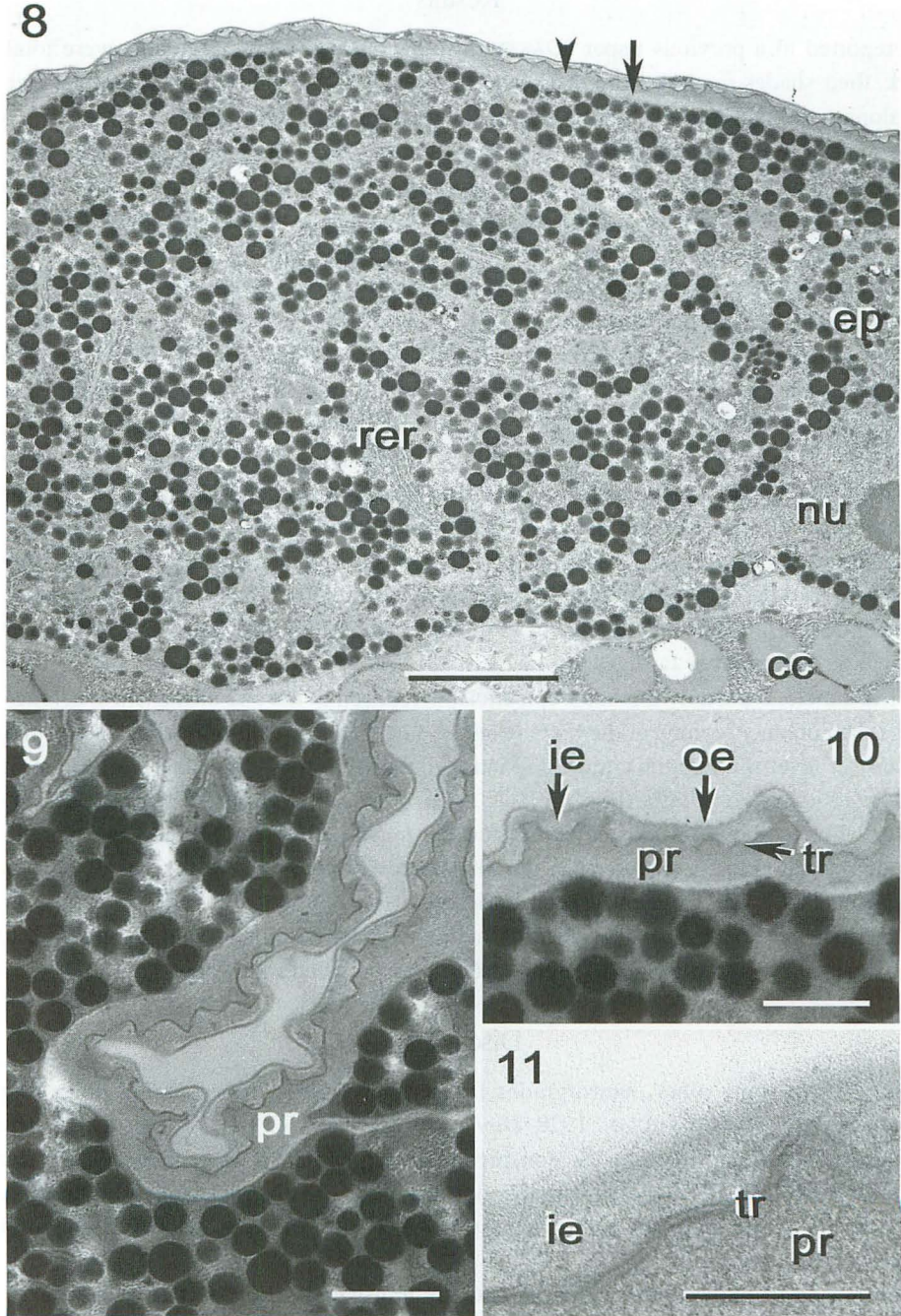
Thickness of the cuticle ranges from approximately 0.5 to 1.0  $\mu\text{m}$ . The cuticle consists of an outer trilaminar epicuticle, a moderately stained, thicker inner epicuticle, followed by a trilaminar layer and the procuticle. In the latter sometimes a slightly denser distal portion can be distinguished from a sparser (lighter) proximal portion just above the epidermis (Figs 8-11). Such specimens probably prepare for moulting (Fig. 8). Epidermal cells are of variable thickness and interdigitate extensively. They contain the usual cell organelles and membrane systems such as the nucleus, mitochondria, rough endoplasmic reticulum and dictyosomes and the above mentioned electron-dense, largely round pigment granules (diameter up to 0.3, rarely 0.5  $\mu\text{m}$ : Figs 8-11, 13). The electron-density of these organelles did not allow to distinguish a surrounding membrane. Abundant light granules of unknown origin and function occur in the body cavity (e.g., Figs 5, 6, 7, 13). They are known also from other tardigrades.

The cuticle, especially the inner epicuticle, is reduced at the site of muscle attachments more than in other regions of the body (Figs 12-14). Here, cuticular fibres extend from the trilaminar layer through the cuticle and intracellular fibres pass the underlying epidermis cell up to its tortuous basis (Figs 13, 14). A thick layer of amorphous material seems to occur between the basal plasmalemma and the muscle. Pigment granules are absent in the epidermal cell associated with the muscle cell. However, expansions of adjacent pigmented cells are frequently seen underlying the pigment-free cell (Fig. 14). The expansions approach the muscle cell and surround the distal end of the muscle attachment, leaving only a small pigment-free area (a “window”: Figs 12, 14).

## Discussion

Compared to some other eutardigrades facultatively living in cryoconite holes (e.g., *Isohypsibius granulifer* THULIN, 1928, *Diphyscon recamierei* RICHTERS, 1911, *D. scoticum* MURRAY, 1905) and even to the conspicuously pigmented *R. oberhaeuseri*, the dark body colour of *H. klebelsbergi* is strikingly intense. It obviously depends on the age of the animals as the pigmentation is lacking in hatchlings and only poorly developed in juveniles (DASTYCH et al. 2003).

As judged from the ultrastructure and totally transparent exuviae of *H. klebelsbergi* – the latter have been repeatedly observed in culture dishes – the cuticle does not or insignificantly contribute to the body colour. The body colour derives from an intensive pigmentation of the epidermis, which completely screens the tissue underneath. The nature of this pigment is unknown; it might be melanin, a substance widespread in invertebrates, including arthropods.



**Figs 8-11.** TEM of the integument of *Hypsibius klebelsbergi* MIHELČIĆ: **8**, within the procuticle a somewhat denser (arrowhead) and a lighter portion (arrow) is seen; note the abundant rough endoplasmic reticulum (*rer*) in the epidermal cell in the specimen that probably prepares for moulting; **9**, the procuticle (*pr*) is rather homogeneous; **10**, cuticle with outer epicuticle (*oe*), inner epicuticle (*ie*), trilaminar layer (*tr*), procuticle (*pr*); **11**, detail of the trilaminar layer (*tr*), inner epicuticle (*ie*), and procuticle (*pr*). (Bar – Fig. 8: 2  $\mu$ m, Figs 9, 10: 1  $\mu$ m, Fig. 11: 0.3  $\mu$ m).

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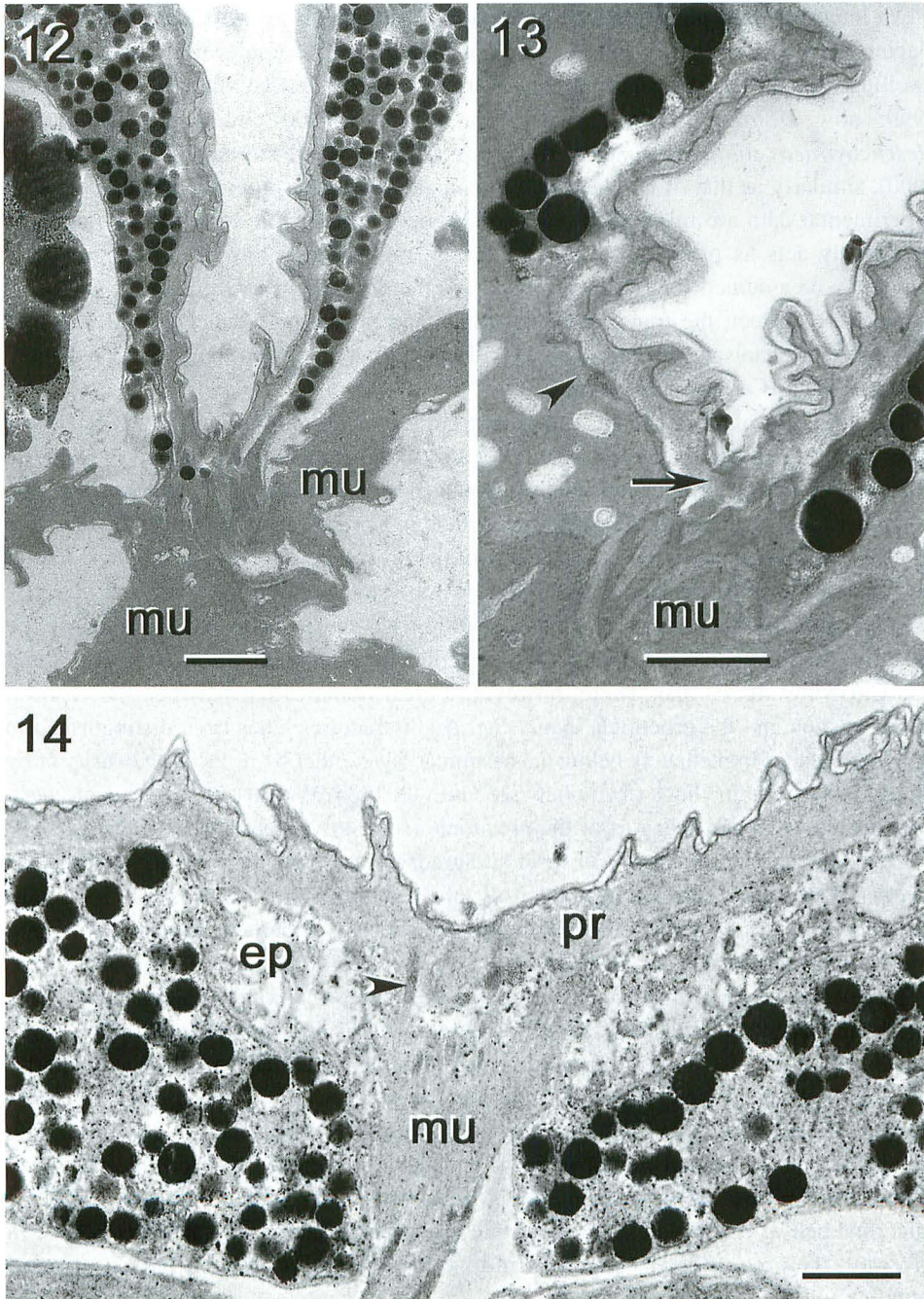
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**Figs 12-14.** TEM of the muscle attachment sites of *Hypsibius klebelsbergi* MIHELČIĆ: **12**, low power micrograph; note the largely pigment free muscle attachment site; muscle (*mu*); **13**, note fibres in the procuticle (arrow) and the extremely thin, pigment-free epidermal cell (arrowhead); **14**, pigmented epidermal cells extend under the pigment-free epidermal cell (*ep*) and surround the muscle (*mu*); note fibres in the procuticle extending in the epidermal cell (arrowhead). The epicuticle is markedly reduced (compare Figs 9, 10); procuticle (*pr*). (Bars – Figs 12-14: 1  $\mu$ m).



An intensive dark pigmentation is known in a variety of invertebrates inhabiting the glaciers' surface and exposed to high UV-radiation. The epidermis of the glacier-dwelling Collembola (the genus *Isotoma* BOURLET, 1839: see EISENBEIS & MEYER 1986, 1999; now *Desoria* NICOLET, 1841: see POTAPOW 2001), and the enchytraeid *Mesenchytraeus solifugus* (EMERY, 1898) (see GOODMAN & PARRISH 1971, SHAIN et al. 2000), similarly as that of *H. klebelsbergi*, contains abundant pigment granules. Though experimental data are missing to our knowledge, the body colouring of these animals supposedly acts as protection against the high radiation on the glacier surface. The same may be assumed for *H. klebelsbergi* (see the discussion in DASTYCH et al. 2003). If this were correct, the transparent hatchlings and poorly pigmented juveniles should have other mechanisms (perhaps behavioural ones) to escape from the harmful radiation. However, details are entirely unknown.

The structure of the delicate transparent cuticle of *H. klebelsbergi* is "typical" for most Eutardigrada (for review see GREVEN 1984) and shows (from outside to inside) a trilaminar epicuticle, the inner epicuticle (reduced markedly at muscle attachments), the trilaminar layer (typical for the tardigrade cuticle in general) and the procuticle. With the fixatives applied herein the electron density of the procuticle did only slightly decrease, if at all, towards the epidermis, but does not allow for distinction of a so-called "intracuticle". Only in animals that obviously started moulting we saw two distinct portions of the procuticle and abundant rough endoplasmic reticulum in epidermal cells (see Fig. 8 and also CROWE et al. 1971b). There, however, a clear demarcation between the electron denser portion and the procuticle is missing. An "intracuticle" has been distinguished in many tardigrades immediately below the trilaminar layer either by its electron density or by special preparative methods (for review see GREVEN 1984, WRIGHT 1988). It represents a modified chitin-containing part of the procuticle (see GREVEN & PETERS 1986).

Generally, all epidermal cells of most tardigrades possess pigment granules, but lighter regions of the body indicate lesser density of these organelles. This can be seen very clearly in *R. oberhaeuseri* even by LM where the muscle attachment sites appear largely free of pigments (MARCUS 1929), creating a typical specific pigment pattern.

The muscle attachments of only two tardigrade species with a fairly pigmentation have been examined as yet with TEM. To them belongs *Macrobotus areolatus* MURRAY, 1907 (see CROWE et al. 1971a: in this species we interpret the epidermal vesicles that are partly filled with electron dense material as pigment organelles) and *Macrobotus hufelandi* SCHULTZE, 1834 (see SHAW 1974). In the latter species the epidermal cell associated with the muscle cell is thin and free of pigment granules. This applies also to *H. klebelsbergi*. In this species the pigmented epidermal cells often extend under the pigment-free epidermal cell of the muscle attachment site approaching the muscle and surround it. In this way, the area affected by the harmful UV-radiation is reduced only to a small "window".

Otherwise the muscle attachments of *H. klebelsbergi* correspond to those known from other Eutardigrada and Heteroartardigrada. Generally, cuticular fibres extend from the trilaminar layer or from the outer epicuticle throughout the cuticle into cuticular projections. From these projections dense fibres cross the narrowed epidermal cell and reach the basal plasmalemma. Between the epidermal cell and the muscle cell occurs a

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modified basal membrane (SHAW 1974, for review see GREVEN 1980; for a generalizing diagram see Fig. 20 in GREVEN 1980). In *H. klebelsbergi* the area of muscle attachment shows a remarkable reduction of the inner epicuticle; it may correspond largely to the "articulation zone" described for *M. areolatus* by CROWE et al. (1971a), where the whole cuticle appears to be thinner.

From a morphological point of view, the organisation of the integument of *H. klebelsbergi* described herein is also exhibited by other tardigrade species. However, the striking difference is a large amount of pigment granules, leaving at the muscle attachments only a few, if any, "windows" penetrable for UV-radiation. This may be regarded as a specific form of protective adaptation to the glacier habitat, in particular to the intense, harmful light.

ACKNOWLEDGEMENTS. We are grateful to Dipl.-Biol. B. PELZER (Universität Düsseldorf) for the help with *TEM*. H. D. thanks Univ. Prof. Dr. K. THALER (Institut für Zoologie und Limnologie, Universität Innsbruck) for his arrangements and help with the material collection and Ms R. WALTER (Universität Hamburg) for her assistance with *TEM*. We thank two referees for their valuable remarks. All support for this project from the universities Hamburg and Innsbruck is gratefully acknowledged.

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Received: 31 March 2005; accepted: 27 May 2005.