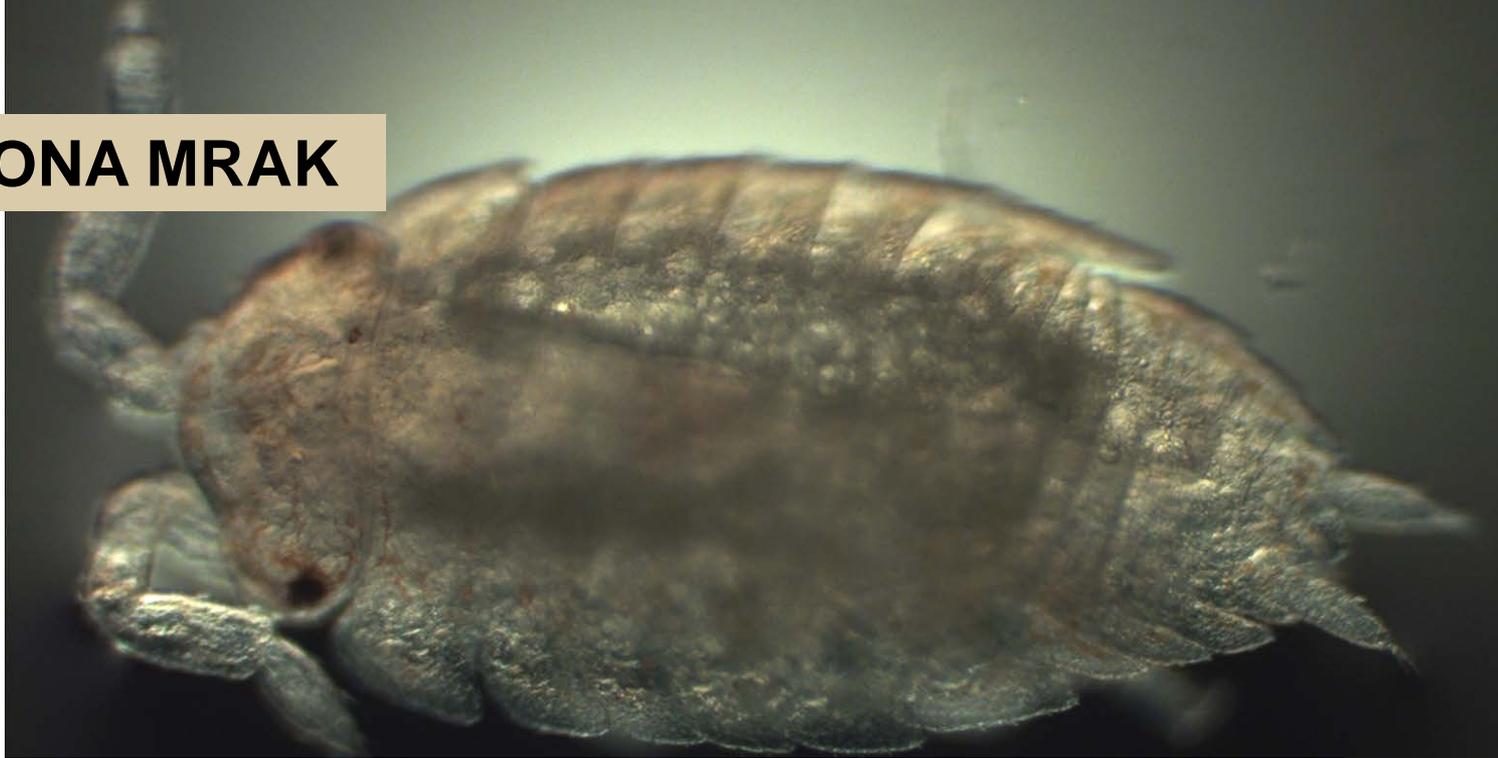


**POLONA MRAK**



## **Tvorba kutikule med razvojem kopenskega raka enakonožca**

**pod mentorstvom  
prof. dr. Jasne Štrus  
doc. dr. Nade Žnidaršič**



## **KUTIKULA**

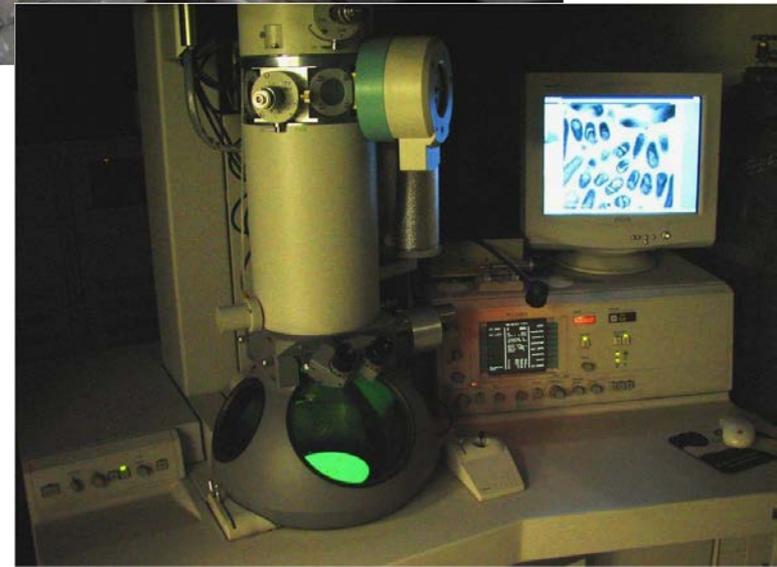
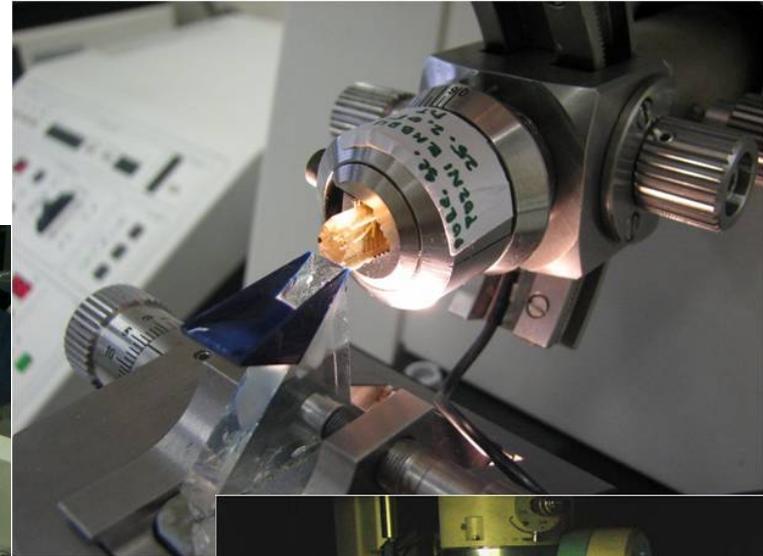
zunani skelet rakov, zunajcelični hitinski matriks epidermalnih celic

## **CELIČNA BIOLOGIJA MINERALIZIRANIH ORGANSKIH MATRIKSOV**

dinamika med nastajanjem in razgrajevanjem

**KOPENSKI RAKI ENAKONOŽCI - Isopoda** (*Porcellio scaber*)  
prilagoditve na kopensko življenje

# Delo v laboratoriju...



SAMICA Z  
MARZUPIJEM



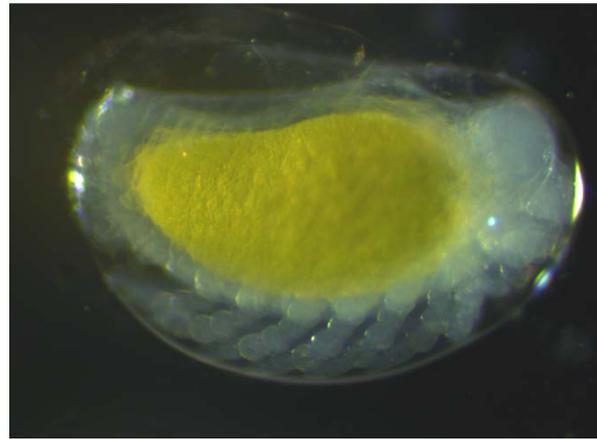
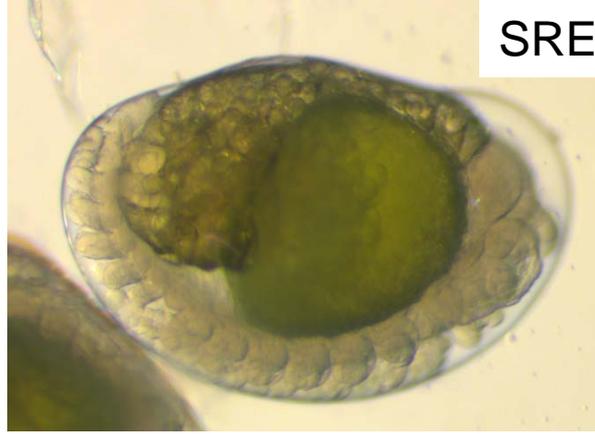
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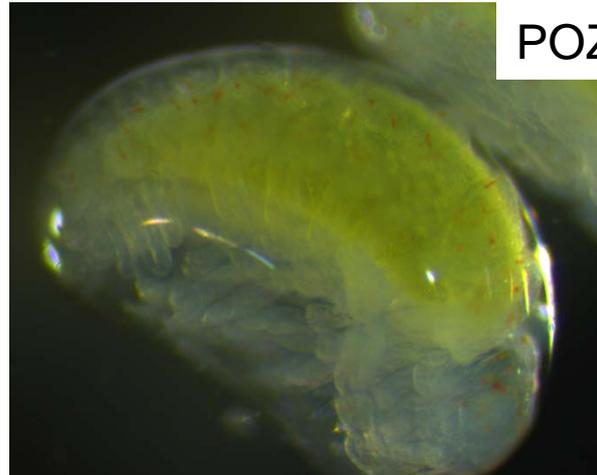
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SREDNJI EMBRIJ



POZNI EMBRIJ



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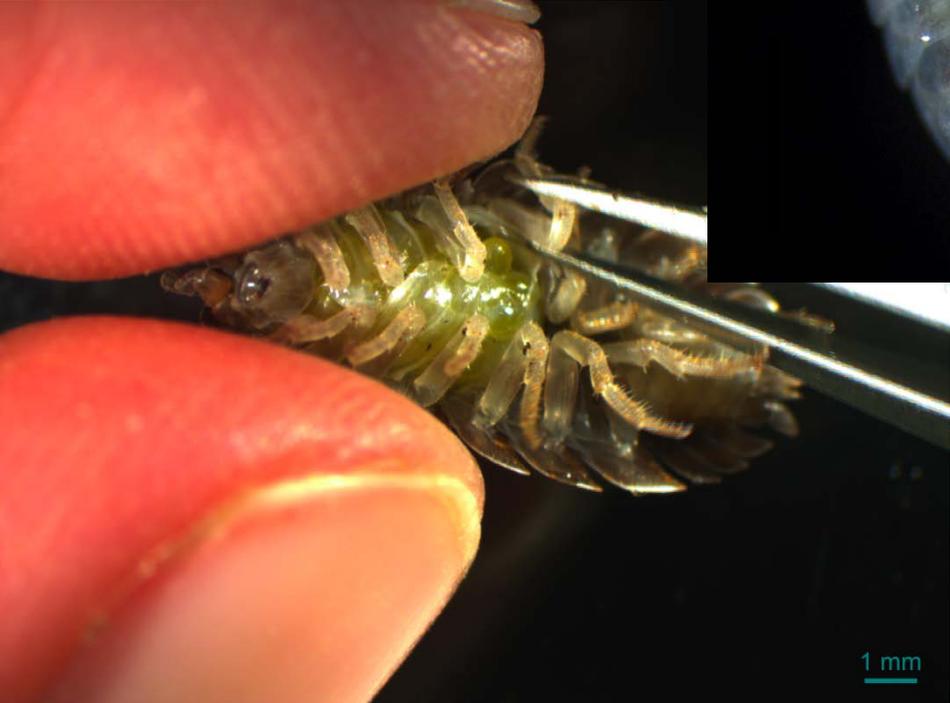
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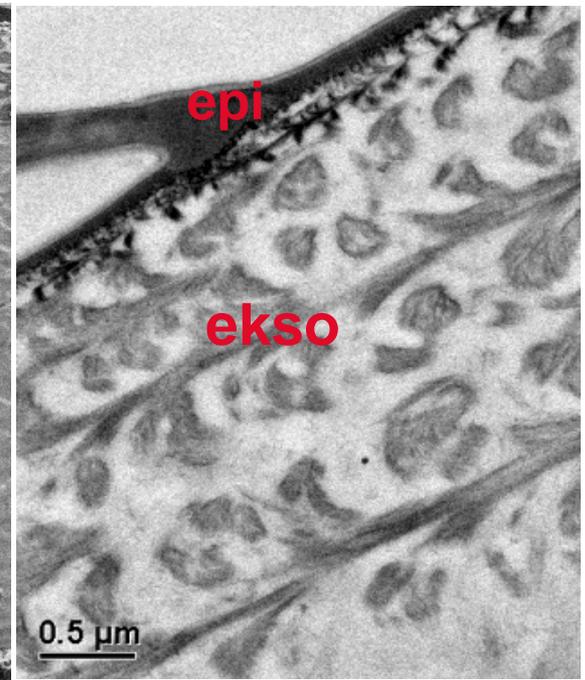
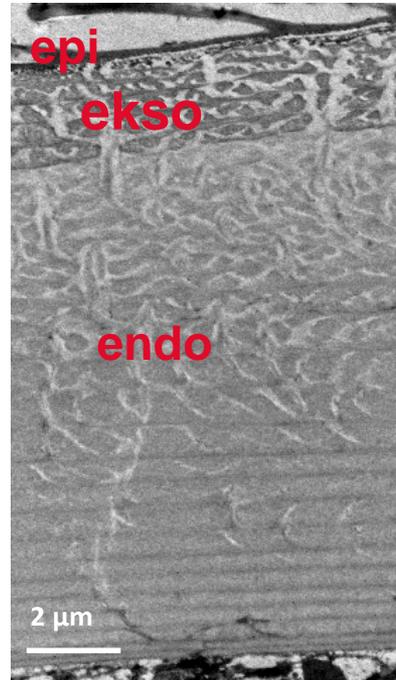
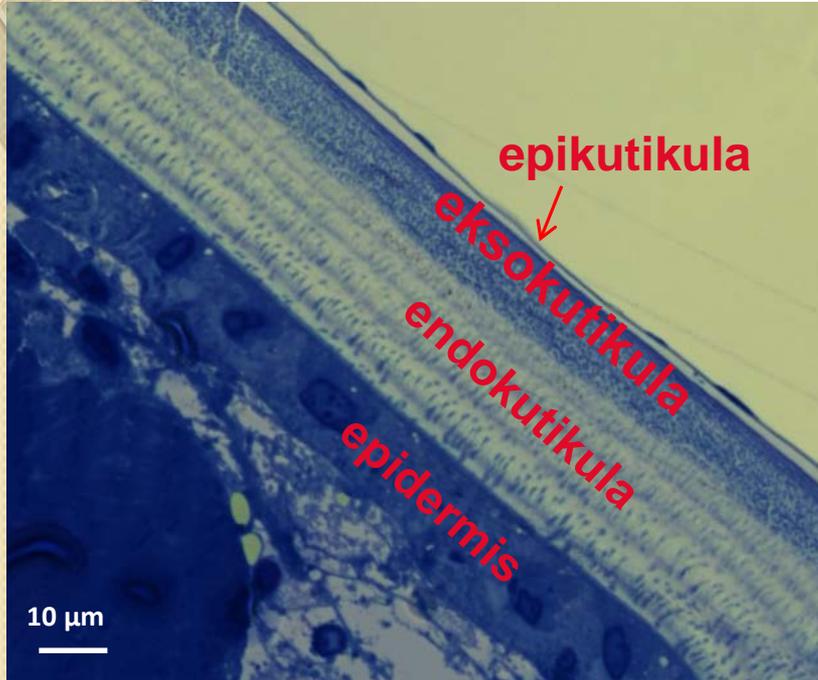
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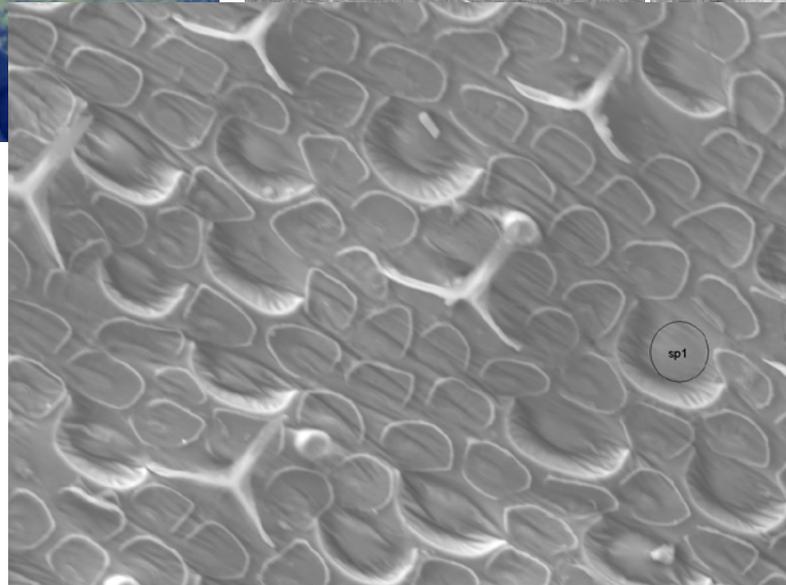
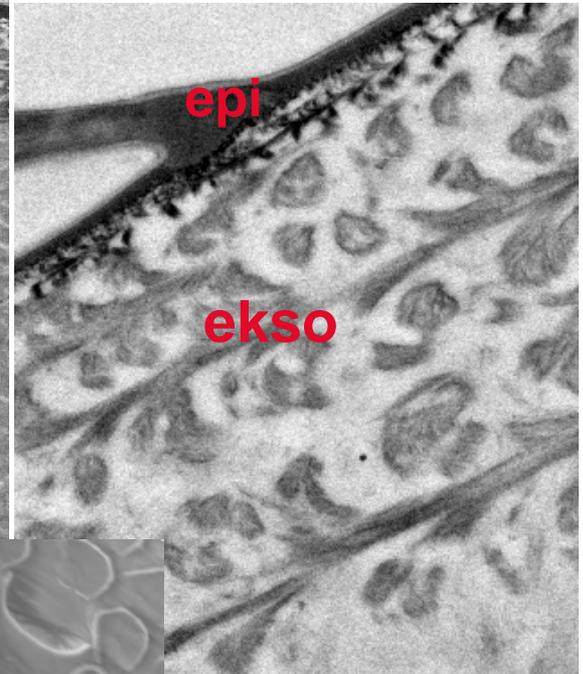
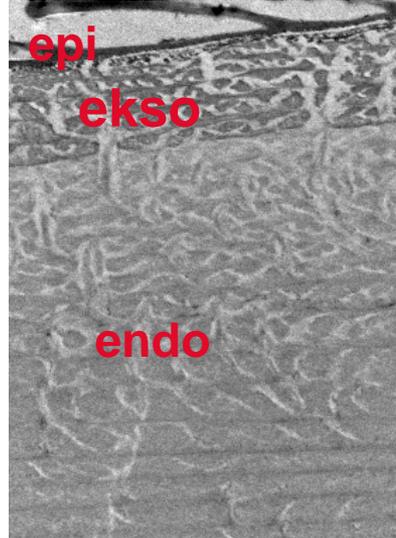
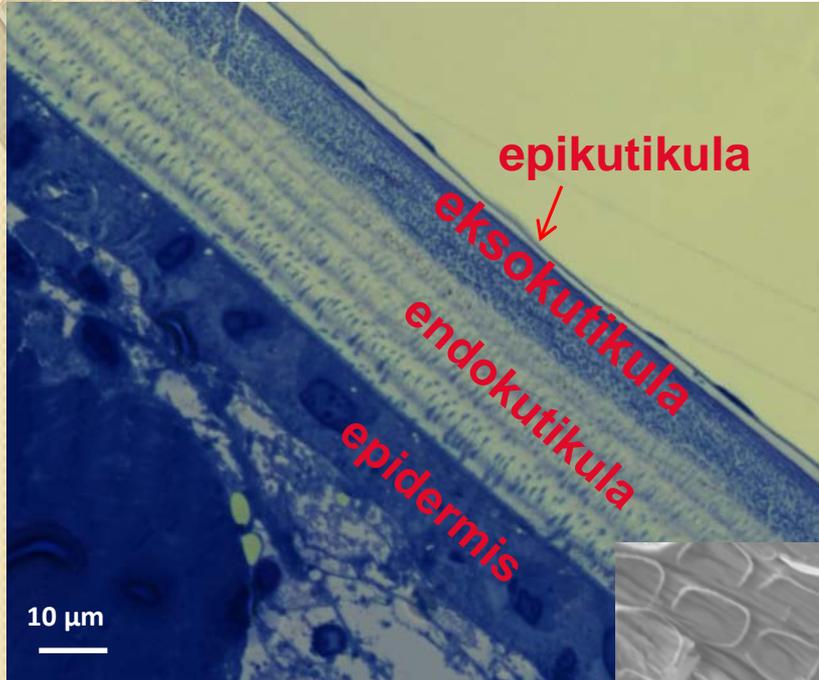
# Mikroskopija

Kutikula odraslega *P. scaber*



# Mikroskopija

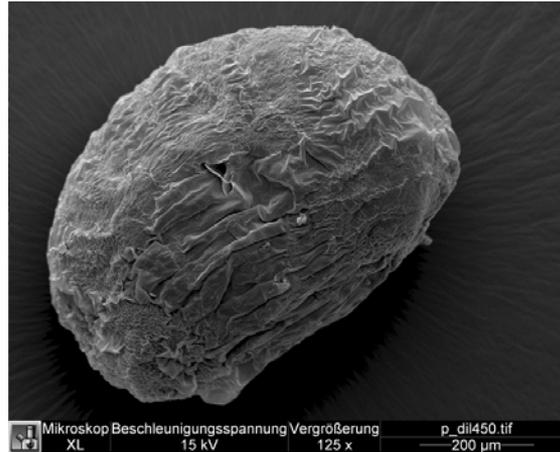
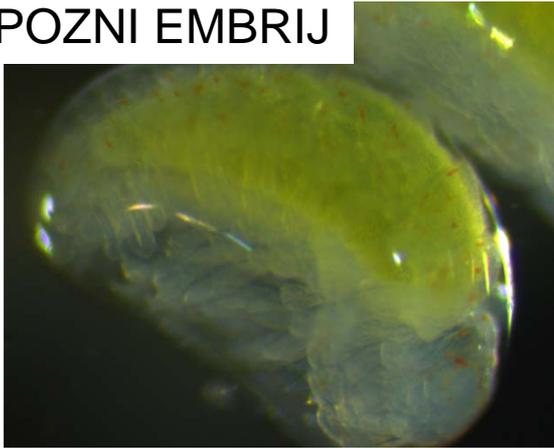
Kutikula odraslega *P. scaber*



X 800 15.0kV LEI SEM WD 15.1mm 10  $\mu\text{m}$  IJS

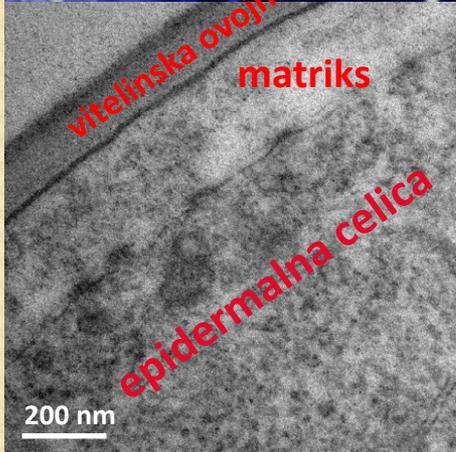
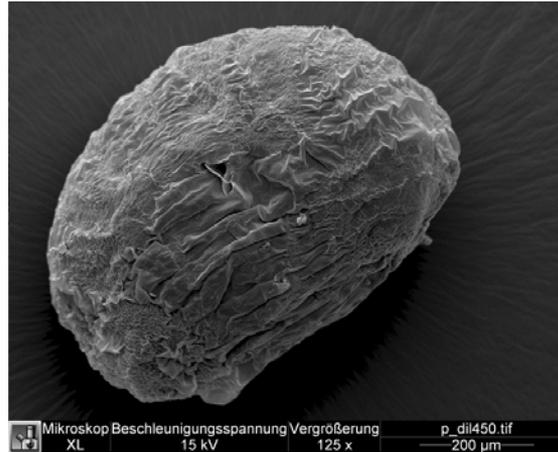
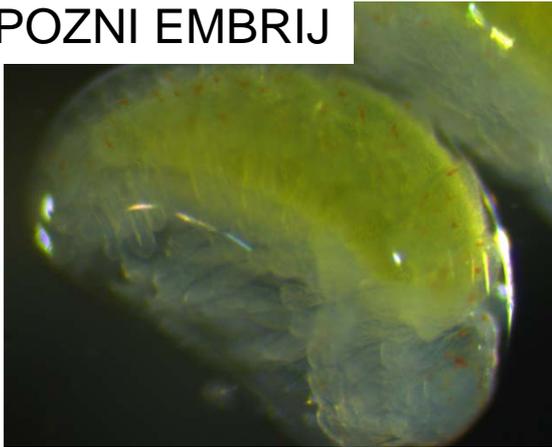
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POZNI EMBRIJ



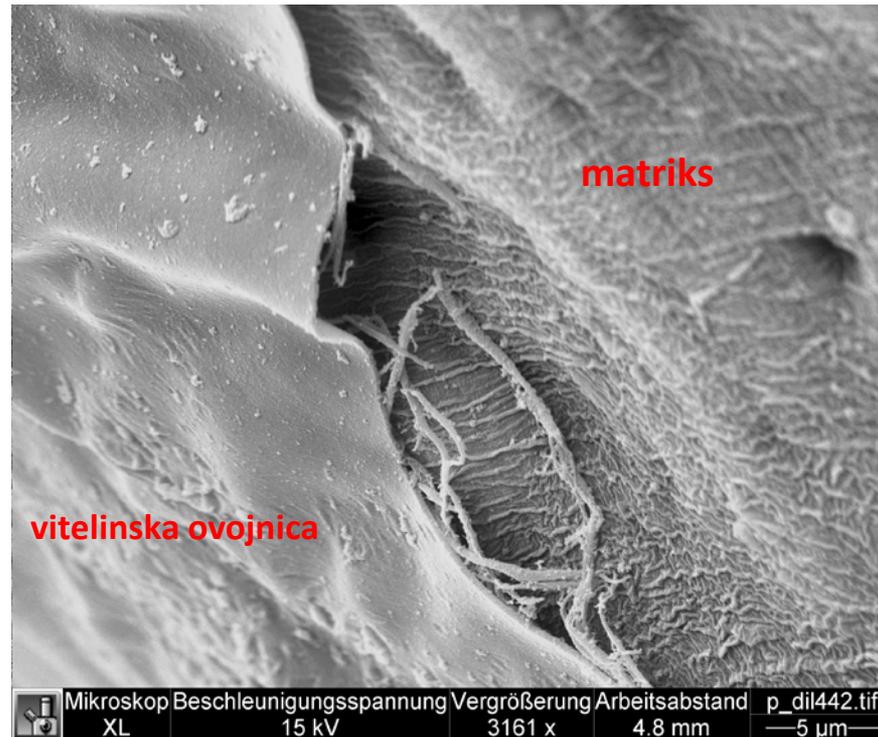
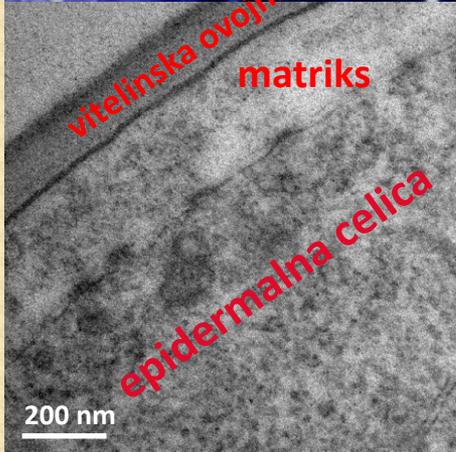
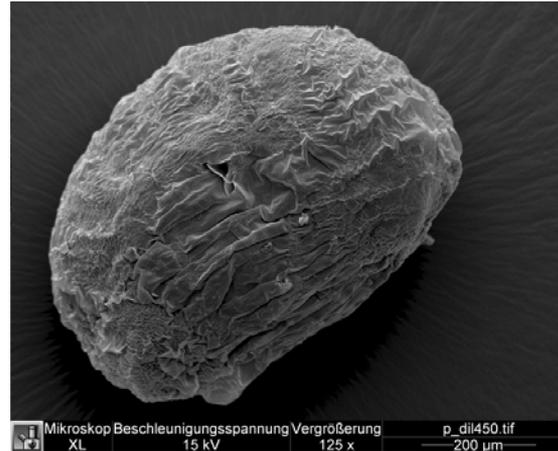
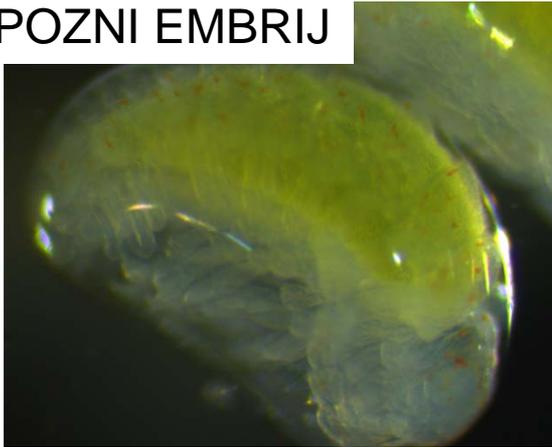
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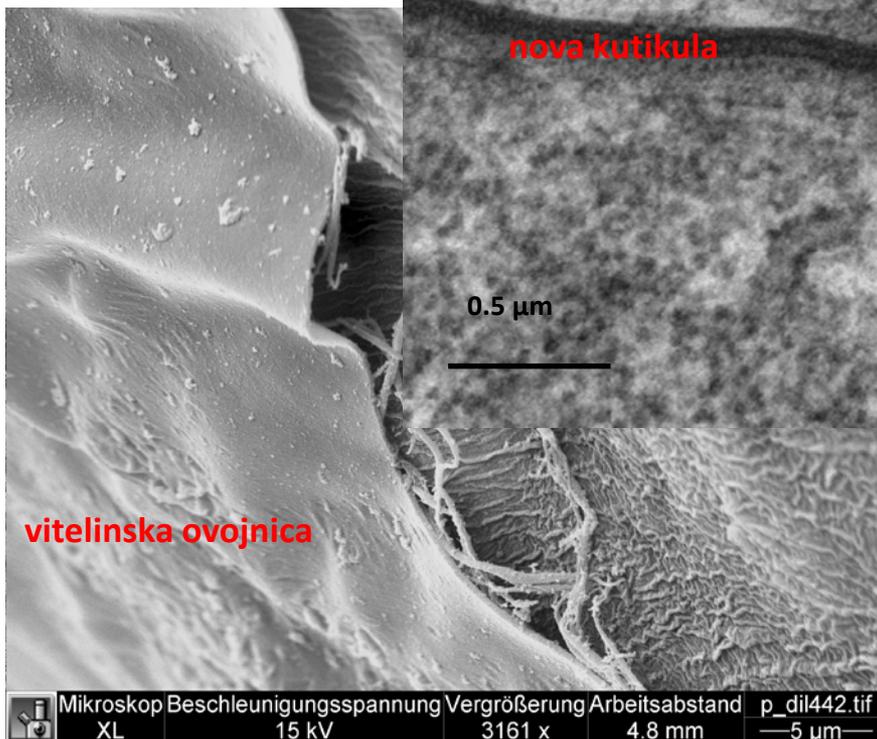
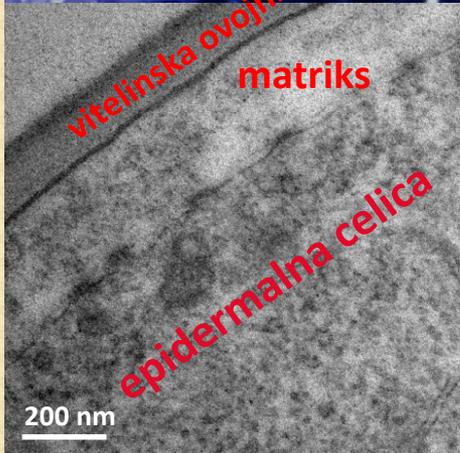
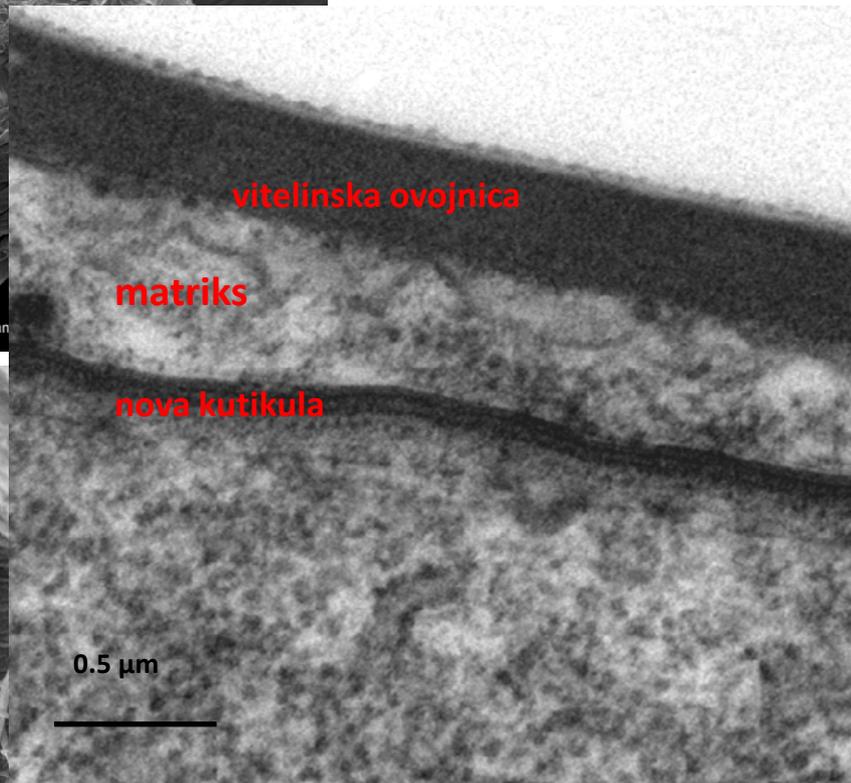
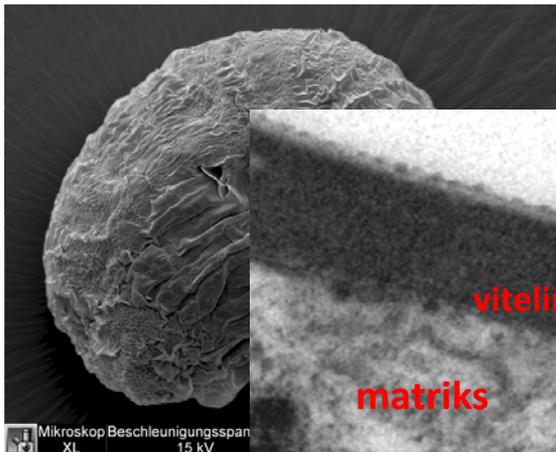
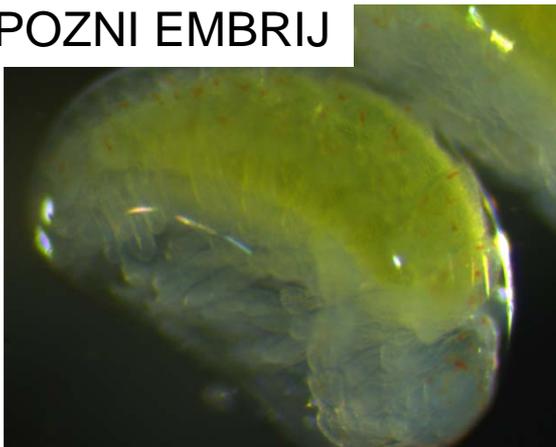
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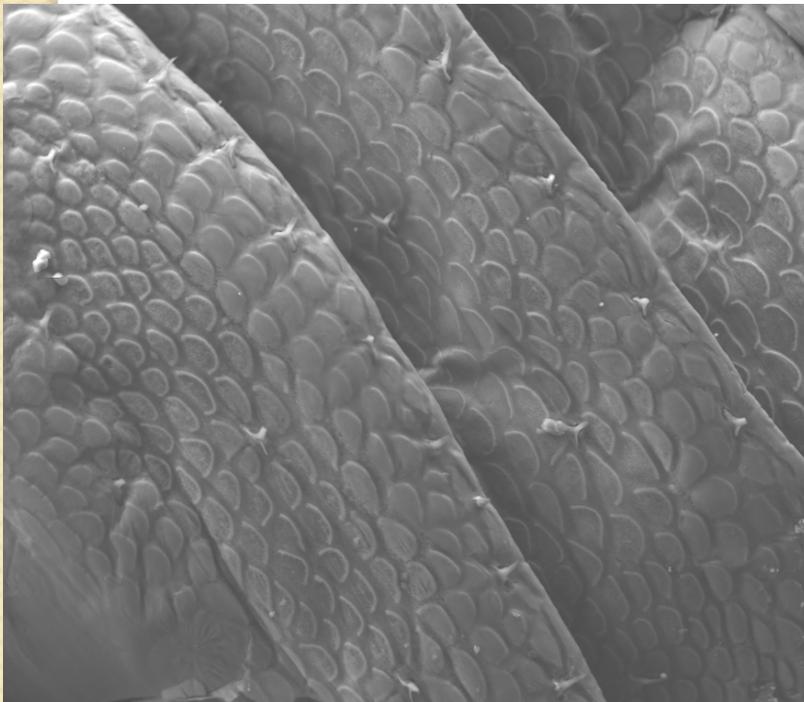
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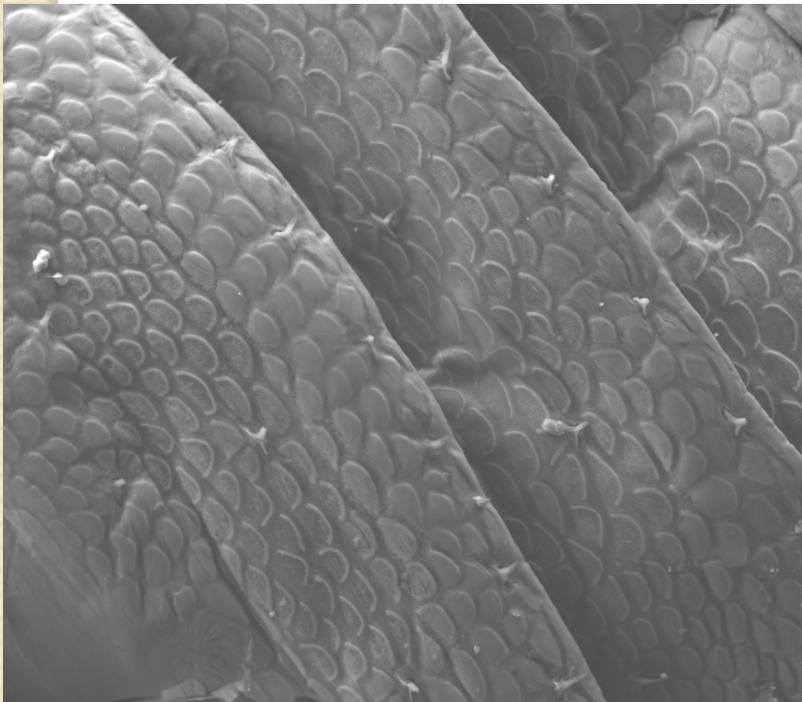
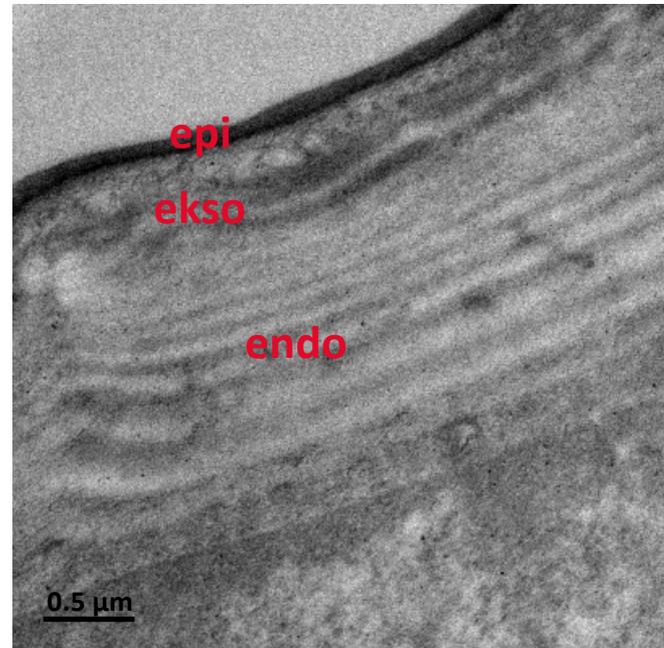
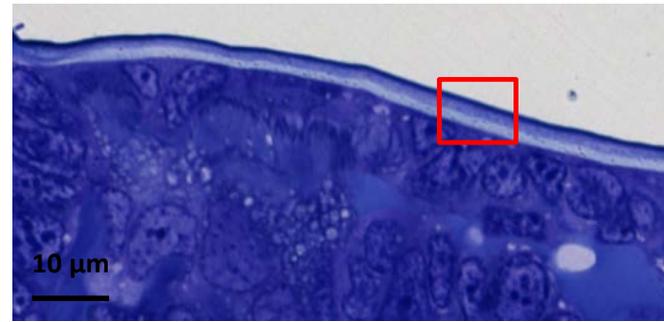
# Mikroskopija

MARZUPIJSKA LARVA  
MANKA



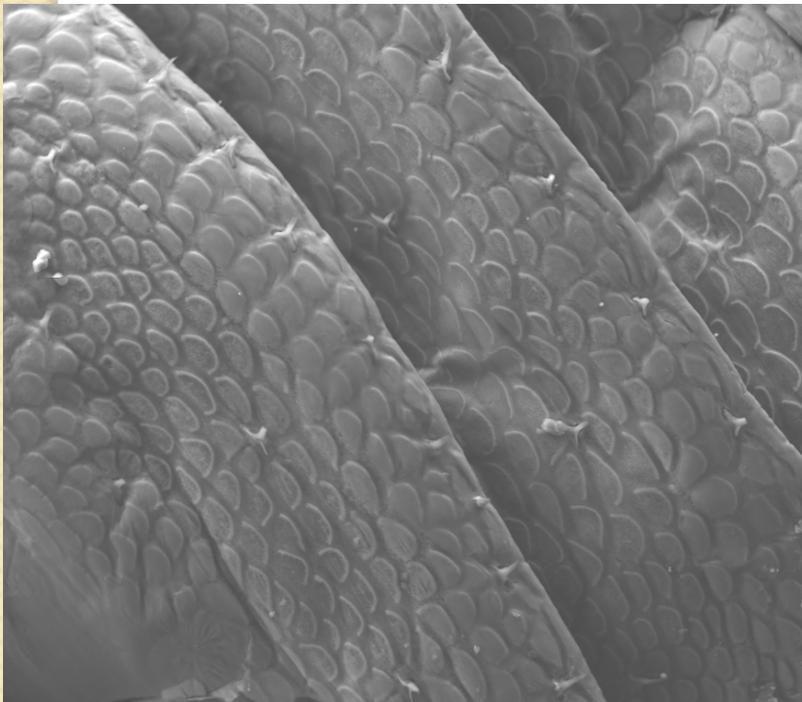
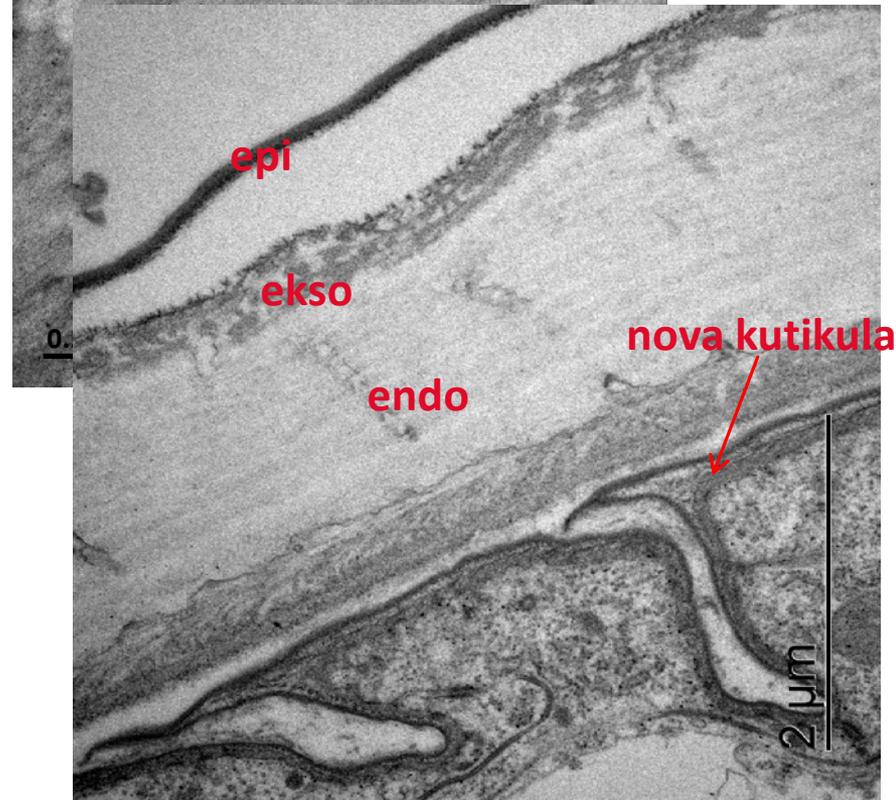
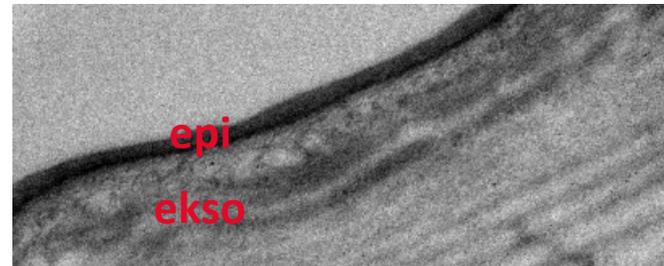
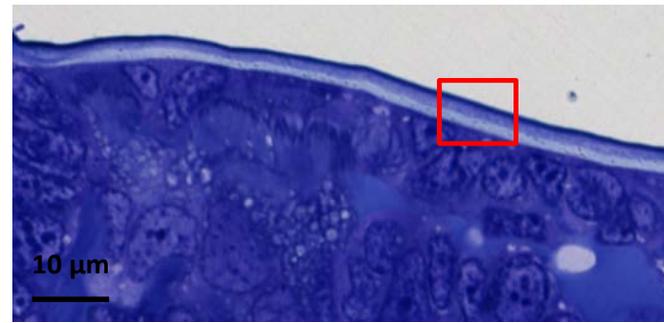
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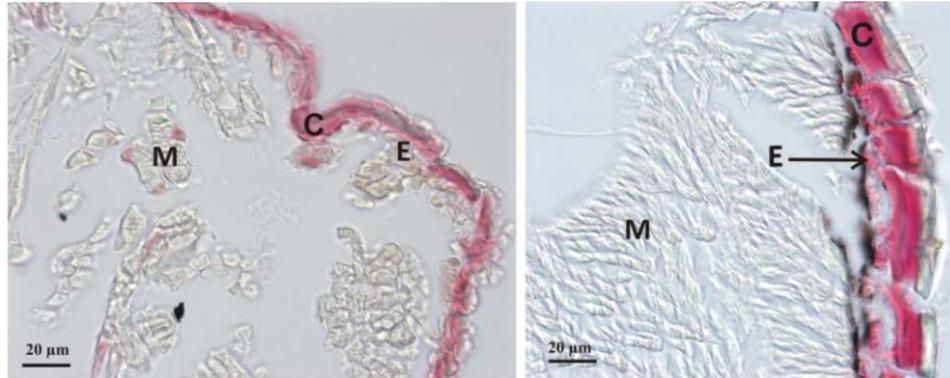
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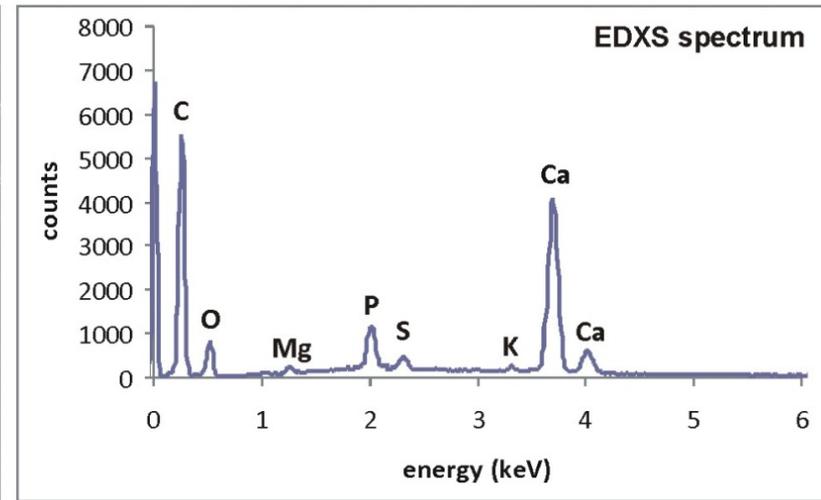
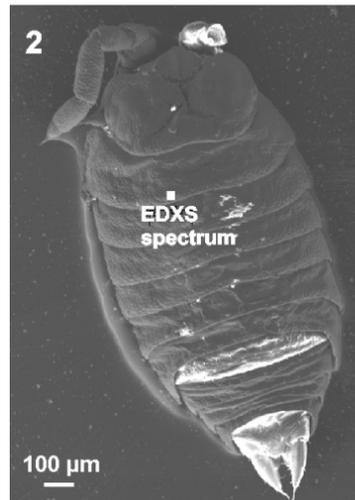


# Analize, lokalizacije

Histokemija:  
alizarin rdeče S



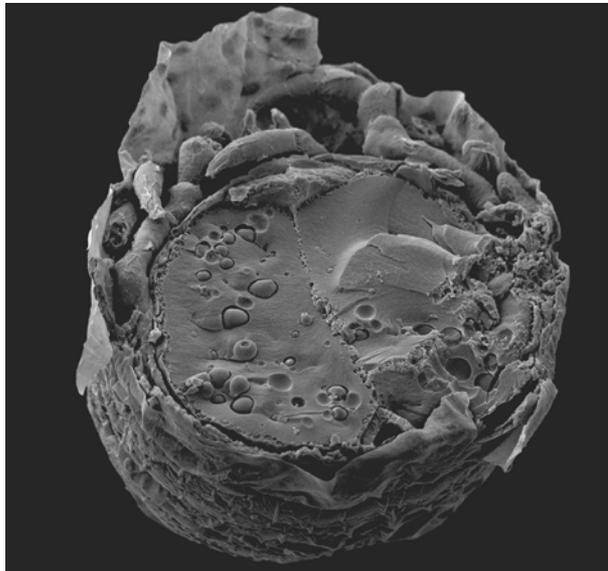
Analitska mikroskopija - sodelovanje z IJS



Lokalizacija hitina, imunolokalizacije celičnih komponent...

# Kratek obisk na Dunaju

Department of Ultrastructural Research, University of Vienna



Mikroskop Beschleunigungsspannung Vergrößerung  
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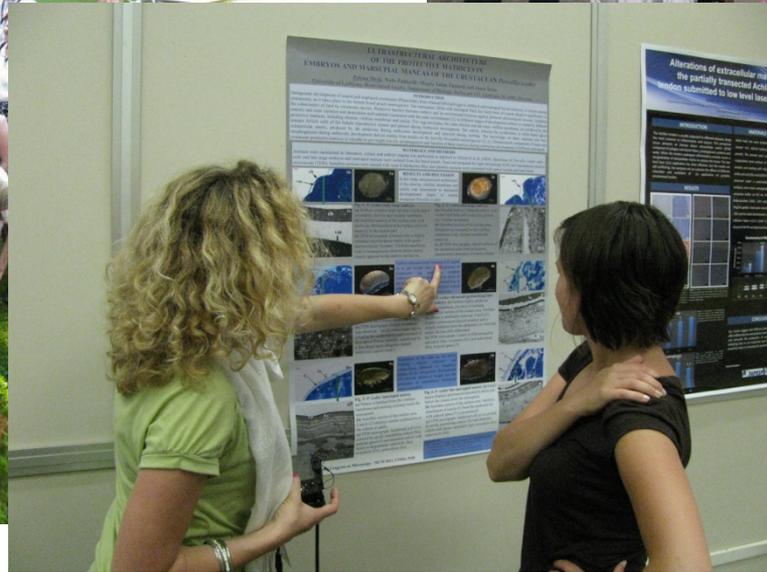
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# Kongresi



8th International  
Symposium of  
Terrestrial Isopod  
Biology 2011,  
Bled

# Kongresi



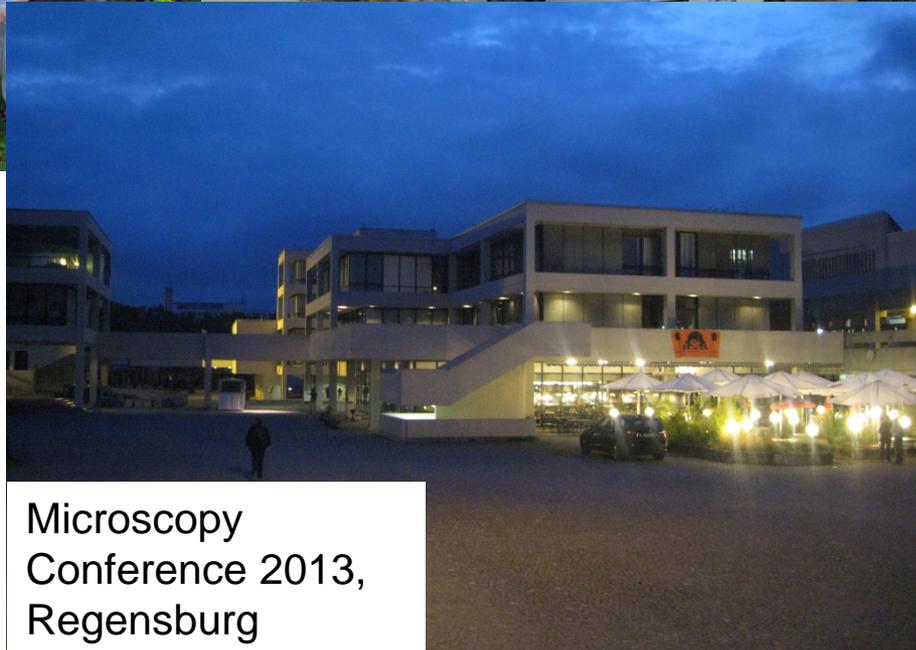
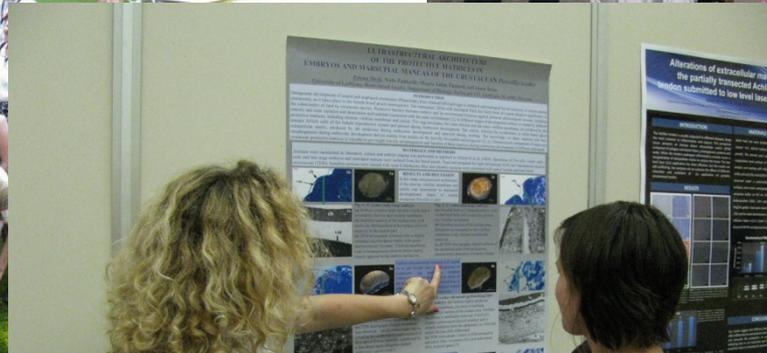
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8th International  
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Biology 2011,  
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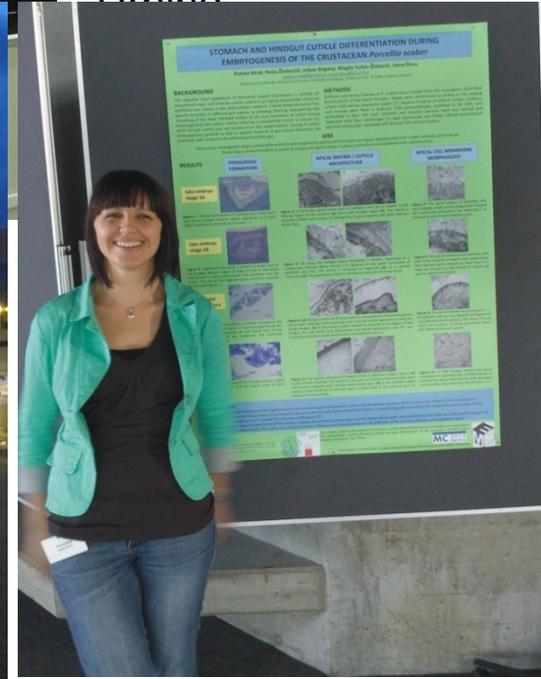
# Kongresi



10th Multinational  
Congress on  
Microscopy 2011,  
Urbino



Microscopy  
Conference 2013,  
Regensburg



8th International  
Symposium of  
Terrestrial Isopod  
Biology 2011,  
Bled

# EMBO tečaj – EM in stereologija v celični biologiji (Češke Budejovice)



# EMBO tečaj – EM in stereologija v celični biologiji (Češke Budejovice)



# EMBO tečaj – EM in stereologija v celični biologiji (Češke Budejovice)



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# EMBO tečaj – EM in stereologija v celični biologiji (Češke Budejovice)



# EMBO tečaj – EM in stereologija v celični biologiji (Češke Budejovice)



# Pisanje...



**LOOK AT ALL THIS WORK  
I HAVENT DONE YET**



# Pisanje...



## CUTICLE STRUCTURE IN PORCELLIO EMBRYOS AND

Mrak P.<sup>1</sup>, Žnidarič N.<sup>1</sup>, Tušek-Žnidarič M.<sup>1</sup>, Gruber D.<sup>2</sup>, Klepal W.<sup>2</sup>, Štrus J.<sup>1</sup>  
<sup>1</sup> Department of Biology, Biotechnical faculty, University of Ljubljana, Ljubljana, Slovenia.  
 E-mail: polonamrak@biol.uni-lj.si, jana.strus@biol.uni-lj.si  
<sup>2</sup> Department of Ultrastructural Research, University of Vienna, Vienna, Austria.

**ABSTRACT.** Crustacean exoskeleton is a chitin-based extracellular matrix, produced in epithelium during embryogenesis and during molting. Data on terrestrial crustacean cuticle embryonic development are very scarce. In this study we report on ultrastructural characteristics of cuticle of late embryos and marsupial manca was already differentiated in three main exocuticle and endocuticle, and displayed architecture similar to the cuticle of adults. Analytically earlier embryonic stages is underway to gain additional insight into crustacean cuticle embryogenesis.

**Keywords:** cuticle, embryo, isopod, ultrastructure

### 1. Introduction

Cuticle is an apical extracellular matrix, comprising distinct horizontal layers, differing in structure and composition. It is produced by the integument, hindgut and foregut epithelia during embryonic development and it is renewed periodically during molting. Cuticle synthesis implies extensive polarized secretion at the apical side of epithelial cells and sequential elaboration of cuticular layers. The knowledge on cuticle differentiation during embryonic development derives mainly from studies on the fruit fly *Drosophila melanogaster*, as this model species offers wide opportunities for a genetic approach to address the cuticle

0.1M cacodylate buffer (pH7.2) the vitelline membrane of emb performed with a thin needle. / cacodylate buffer, the samples w osmium tetroxide for 2 hours, dehydrated. Specimens for IM and TEM Agar 100 resin. Prior to embed performed with a thin needle for resin. Semithin sections were cut stained with Azur II-Methylene 1 Zeiss AxioImager Z.1 light m sections were cut by a Re ultramicrotome (Leica), contrast osmium and 1% lead citrate, an

## Cuticle formation during marsupial devel

### Porcellio scaber: Imaging and analysis

P. Mrak<sup>1</sup>, N. Žnidarič<sup>1</sup>, K. Žagač<sup>2</sup>, M. Čeh<sup>2</sup>, J. Štrus<sup>1</sup>

<sup>1</sup> Department of Biology, Biotechnical faculty, University of Ljubljana, Večna pot 2, Department for Nanostructured Materials, Jožef Stefan Institute, Jamova cest

polona.mrak@bi.uni-lj.si

Keywords: embryo, marsupial manca, terrestrial isopods, exoskeleton,

**Introduction:** Crustacean exoskeleton is a chitin-protein matrix with epidermis. Organic cuticle constituents are hierarchically ordered minerals, mostly calcium carbonate and phosphate. The data outline during embryogenesis provide knowledge of cell biology biomineralization processes and the role of hard biologic intramarsupial developmental stages of terrestrial isopod *Porcellio scaber* of cuticle ultrastructure, localization of N-acetylglucosamine of mineral component. The embryonic development of terrestrial environment of the female marsupium. Embryos hatch into mar marsupium for another week. Our results are compared with *Drosophila melanogaster* with non-mineralized exoskeleton and aquatic amphipod crustacean *Parhyale hawaiiensis* [3].

**Methods:** A detailed staging system through twenty pr characterize the stages of *P. scaber* embryos and man. Conventional ultrastructural study and labelling of N-acetyl performed on ultrathin sections. The elemental composition of t fixed specimens by energy-dispersive x-ray spectroscopy (EDX). Results and discussions: Ultrastructural and compositional s matrices was performed in two sequential stages of late emb

## Egg envelopes and cuticle renewal in Porcellio embryos and marsupial manca

Polona Mrak<sup>1</sup>, Nada Žnidarič<sup>1</sup>, Magda Tušek-Žnidarič<sup>1</sup>, Waltraud Klepal<sup>2</sup>, Daniela Gruber<sup>2</sup>, Jasna Štrus<sup>1</sup>

<sup>1</sup> Department of Biology, Biotechnical faculty, University of Ljubljana, Večna pot 111, SI-1000 Ljubljana, Slovenia; <sup>2</sup> Department of Ultrastructural Research, University of Vienna, Althanstrasse 14, A-1050 Vienna, Austria

Corresponding author: Polona Mrak (polona.mrak@bi.uni-lj.si)

Academic editor: S. Sjöman (sjo@zoology.gu.se) | Received 22 November 2011 | Accepted 26 January 2012 | Published 09 February 2012

Citation: Mrak P, Žnidarič N, Tušek-Žnidarič M, Klepal W, Gruber D, Štrus J (2012) Egg envelopes and cuticle renewal in *Porcellio* embryos and marsupial manca. ZooKeys 55: 1–12. doi: 10.3897/zookeys.55.2418

### Abstract

An important adaptation to land habitats in terrestrial isopod crustaceans is development of embryos in a fluid-filled female brood pouch, marsupium. The study brings insight into the structure and protective role of egg envelopes and cuticle renewal during ontogenetic development of *Porcellio* embryos and marsupial manca. Egg envelopes cover embryos, the outer chorion until late-stage embryo and the inner vitelline membrane throughout the whole embryonic development. Egg envelopes of *Porcellio* have relatively simple ultrastructural architecture compared to *Drosophila* egg envelopes. Exoskeletal cuticle is produced in late embryonic development by hypodermal cells of the embryo and is renewed in further development in relation to growth of developing embryos and manca. Cuticle structure and renewal in prehatching late-stage embryos and marsupial manca exhibit main features of cuticle in adults. Epicuticle is thin and homogenous, the characteristic arrangement of chitin-protein fibers and the dense dextral layer in exocuticle are hardly discernible in prehatching embryo and distinct in marsupial manca. Endocuticle consists of alternating electron dense and electron lucent sublayers and is perforated by pore canals in both stages. Differences from adult cuticle are evident in cuticle thickness, ultrastructure and mineralization. Signs of cuticle renewal in prehatching embryo and marsupial manca such as detachment of cuticle from hypodermis, partial disintegration of endocuticle and assembly of new cuticle are described.

### Keywords

chorion, vitelline membrane, cuticle, molting, ontogenetic development, terrestrial isopods

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## CUTICLE STRUCTURE IN Porcellio EMBRYOS

Mrak Polona (1), Žnidarič Nada (1), Tušek-Žnidarič Magda (1), Gruber Daniela (2), Klepal Waltraud (2), Štrus Jasna (1)  
<sup>1</sup> University of Ljubljana, Biotechnical faculty, Department of Biology, Ljubljana  
<sup>2</sup> University of Vienna, Department of Ultrastructural Research, Vienna

### INTRODUCTION

Crustacean exoskeleton is a chitin-based extracellular matrix, produced by the hypodermal epithelium during embryonic and during molting. Cuticle synthesis implies extensive polarized secretion at the apical side of epithelial cells sequential elaboration of cuticular layers. The knowledge on cuticle differentiation during embryonic development is mainly from studies on the fruit fly *Drosophila melanogaster* (Mönnich, 2010). Insect and crustacean exoskeleton are of the same common features regarding overall cuticle architecture and chitin-based structural framework, but it has not been considered in comparison. Data on terrestrial crustacean cuticle differentiation during embryonic development are very scarce. To our knowledge only one terrestrial species has been studied from this respect recently (Hlaváčková 2008). In this study we report on ultrastructural characteristics of the exoskeletal cuticle in three developmental stages within the brood pouch of isopod crustacean *Porcellio scaber* females (Fig. 1, 2, 3).

### MATERIALS AND METHODS

Animals were maintained in la reported in Malinve et al. (2009), but cuticle differentiation was not followed in detail. Here we present preliminary results of the exoskeletal cuticle ultrastructure in two stages of late embryos (Fig. 1, 2) and in marsupial manca (Fig. 3). Late embryonic epidermis is covered by disordered fibrous material, supported by a thin dense layer, altogether named embryonic cuticle (Fig. 4). The cuticle of prehatching late embryos and marsupial manca was already differentiated in three main layers, epicuticle, exocuticle and endocuticle, and displayed architecture similar to the cuticle of adults (Fig. 5, 6). Manca molt inside the brood pouch and here we present ultrastructural data on degrading and newly assembling cuticle in marsupial manca (Fig. 6).

### RESULTS AND DISCUSSION

*Porcellio scaber* ontogenetic development in the brood pouch, from released fertilized egg to marsupial manca, was described morphologically (Malinve et al., 2010; Watt, 2009), but cuticle differentiation was not followed in detail. Here we present preliminary results of the exoskeletal cuticle ultrastructure in two stages of late embryos (Fig. 1, 2) and in marsupial manca (Fig. 3). Late embryonic epidermis is covered by disordered fibrous material, supported by a thin dense layer, altogether named embryonic cuticle (Fig. 4). The cuticle of prehatching late embryos and marsupial manca was already differentiated in three main layers, epicuticle, exocuticle and endocuticle, and displayed architecture similar to the cuticle of adults (Fig. 5, 6). Manca molt inside the brood pouch and here we present ultrastructural data on degrading and newly assembling cuticle in marsupial manca (Fig. 6).

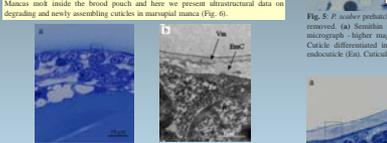


Fig. 1. *P. scaber* late embryo. (a) Semithin section of the surface area. (b) TEM micrograph - higher magnification of the squared area in (a); embryonic cuticle (EmC) between the vitelline membrane (Vm) and epidermal cells (Ec).

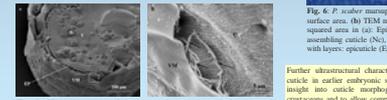


Fig. 2. *P. scaber* late embryo. (a) Semithin section of the surface area. (b) TEM micrograph - higher magnification of the squared area in (a); embryonic cuticle (EmC) between the vitelline membrane (Vm) and epidermal cells (Ec).

## CALCIFICATION OF LARVAL AND POSTMO CRUSTACEANS AS REVEALED BY EDX

Polona Mrak<sup>1</sup>, Nada Žnidarič<sup>1</sup>, Kristina

<sup>1</sup> Department of Biology, Biotechnical faculty, University of Ljubljana, Večna pot 2, Department for Nanostructured Materials, Jožef Stefan Institute, Jamova cest

### INTRODUCTION

Crustacean exoskeletal cuticle is an epidermal apical extracellular matrix, based on chitin-protein fibers. Mature cuticle in adult isopod crustaceans is mineralized by calcite, amorphous calcium carbonate and calcium phosphate (Becker et al., 2005, and et al., 2008, 2009). The cuticle is formed de novo during ontogenetic development in the female brood pouch - marsupium (Mrak et al., 2012) and during molting in adults. Calcification in invertebrates part of cuticle formation, but data on calcification of the forming cuticle is very limited.

### AIM

In this study a combination of microscopic methods was applied to obtain data on ultrastructure and composition of the exoskeletal cuticle in different developmental stages of *Porcellio scaber* marsupial manca, in comparison to adult isopod crustaceans in molting period. Ultrastructure was determined by TEM of glutaraldehyde fixed and resin included embedded tissue ultrathin sections. SEM - EDX analyses were performed in forage method fixed, air dried and carbon coated specimens, for histochemical elemental analysis screening paraffin and cryostat sections were used.

### RESULTS

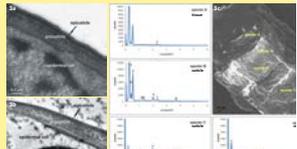


Fig. 3. Early-stage marsupial manca of *P. scaber*, soon after hatching from the vitelline membrane. Epicuticle (e) is up to 1 µm thick and differentiated into ep- and pro-cuticle. SEM-EDX spectra of glutaraldehyde fixed (emerged) (a) and non-removed (b) (a) leg. SEM image of transversely cut manca and positions of EDX analyses. EDX spectra obtained on the manca surface show calcium and phosphorus peaks in the cuticle (spectra B, C and D). The Ca/P ratio is < 1 in all spectra. In addition, magnesium was detected in the cuticle. Specter of the internal tissues does not display Ca and Mg peaks (spectrum A).

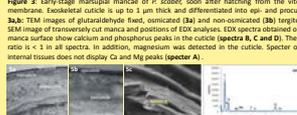


Fig. 4. Advanced early-stage marsupial manca of *P. scaber*. SEM image of the cuticle showing calcium and phosphorus peaks in the cuticle (spectra B, C and D). The Ca/P ratio is < 1 in all spectra. In addition, magnesium was detected in the cuticle. Specter of the internal tissues does not display Ca and Mg peaks (spectrum A).

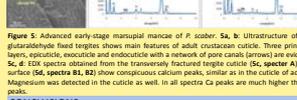


Fig. 5. Marsupial manca of *P. scaber*. SEM image of the cuticle showing calcium and phosphorus peaks in the cuticle (spectra B, C and D). The Ca/P ratio is < 1 in all spectra. In addition, magnesium was detected in the cuticle. Specter of the internal tissues does not display Ca and Mg peaks (spectrum A).

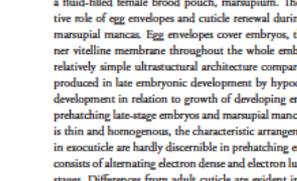


Fig. 6. Marsupial manca of *P. scaber*. SEM image of the cuticle showing calcium and phosphorus peaks in the cuticle (spectra B, C and D). The Ca/P ratio is < 1 in all spectra. In addition, magnesium was detected in the cuticle. Specter of the internal tissues does not display Ca and Mg peaks (spectrum A).



Fig. 7. Marsupial manca of *P. scaber*. SEM image of the cuticle showing calcium and phosphorus peaks in the cuticle (spectra B, C and D). The Ca/P ratio is < 1 in all spectra. In addition, magnesium was detected in the cuticle. Specter of the internal tissues does not display Ca and Mg peaks (spectrum A).

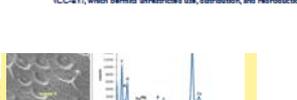


Fig. 8. Marsupial manca of *P. scaber*. SEM image of the cuticle showing calcium and phosphorus peaks in the cuticle (spectra B, C and D). The Ca/P ratio is < 1 in all spectra. In addition, magnesium was detected in the cuticle. Specter of the internal tissues does not display Ca and Mg peaks (spectrum A).

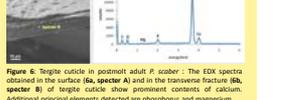


Fig. 9. Marsupial manca of *P. scaber*. SEM image of the cuticle showing calcium and phosphorus peaks in the cuticle (spectra B, C and D). The Ca/P ratio is < 1 in all spectra. In addition, magnesium was detected in the cuticle. Specter of the internal tissues does not display Ca and Mg peaks (spectrum A).

**CONCLUSIONS**  
 In early stage marsupial manca soon after hatching from the vitelline membrane the initial cuticle calcification is indicated as Ca, Mg and P were determined by EDX analysis, but strongly calcified tissue was not evident in histochemical staining. The elemental composition of cuticle in advanced early marsupial manca is similar to that in adult specimens, with prominent Ca, Mg and P peaks. In late stage marsupial manca histochemical staining and ultrastructure reveal that exoskeleton is similar to adults.

## Egg envelopes and cuticle renewal in *Porcellio* embryos and marsupial mancas

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### CUTICLE STRUCTURE IN *PORCELLIO* EMBRYOS AND

Mrak P<sup>1</sup>, Žnidarič N<sup>1</sup>, Tušek-Žnidarič M<sup>1</sup>, Gruber D<sup>2</sup>, Klepač W<sup>2</sup>, Strus J<sup>1</sup>  
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<sup>2</sup> Department of Ultrastructural Research, University of Vienna, Vienna, Austria.

**ABSTRACT:** Crustacean exoskeleton is a chitin-based extracellular matrix, produced in epithelium during embryogenesis and during molting. Data on terrestrial crustacean cuticle embryonic development are very scarce. In this study we report on ultrastructural characteristics of cuticle of late embryos and marsupial mancas was already differentiated in three main exocuticle and endocuticle, and displayed architecture similar to the cuticle of adults. Analytically earlier embryonic stages is underway to gain additional insight into crustacean cuticle embryogenesis.

**Keywords:** cuticle, embryo, isopod, ultrastructure

#### 1. Introduction

Cuticle is an apical extracellular matrix, comprising distinct horizontal layers, differing in structure and composition. It is produced by the integument, hindgut and foregut epithelia during embryonic development and it is renewed periodically during molting. Cuticle synthesis implies extensive polarized secretion at the apical side of epithelial cells and sequential elaboration of cuticular layers. The knowledge on cuticle differentiation during embryonic development derives mainly from studies on the fruit fly *Drosophila melanogaster*, as this model species offers wide opportunities for a genetic approach to address the cuticle

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### Cuticle formation in *Porcellio scaber*: IM

P. Mrak<sup>1</sup>, N. Žnidarič<sup>1</sup>, K. Žač

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2. Department for Nanostructural Materials, University of Vienna, Austria

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**Methods:** A detailed study characterizes the stages of Conventional ultrastructural: performed on ultrathin section fixed specimens by energy-dispersive X-ray (EDX) analysis. Results and discussions: Ultrastructural analysis was performed in th

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Crustacean exoskeletal cuticle is an apical extracellular matrix, produced by the epithelium during embryogenesis and during molting. Cuticle synthesis implies extensive polarized secretion at the apical side of epithelial cells and sequential elaboration of cuticular layers. The knowledge on cuticle differentiation during embryonic development derives mainly from studies on the fruit fly *Drosophila melanogaster* (Mönnich, 2010). Insect and crustacean exoskeletons share some common features regarding overall architecture and chitin-based structural framework, but on the other hand differ considerably in composition. Data on terrestrial crustacean cuticle differentiation during embryonic development are very scarce. To our knowledge only one general study has been published from this respect recently (Bilavston, 2008). In this study we report on ultrastructural characteristics of the exoskeletal cuticle in three developmental stages within the brood pouch of isopod crustacean *Porcellio scaber* females (Fig. 1-3).

#### MATERIALS AND METHODS

**Animals**  
Animals were maintained in laboratory conditions. Late embryos and marsupial mancas were collected from the brood pouches of *Porcellio scaber* females. Animals were stained with Azur II-Methylene I Zeiss AxioImager Z1 light microscope (SEM).

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**TEM**  
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**EDX**  
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### CUTICLE STRUCTURE IN *Porcellio* EMBRYOS

Mrak Polona (1), Žnidarič Nada (1), Tušek-Žnidarič Magda (1), Gruber Daniela (2), Klepač Waltraud (2), Strus Jasna (1)  
(1) University of Ljubljana, Biotechnical faculty, Department of Biology, Ljubljana, Slovenia  
(2) University of Vienna, Department of Ultrastructural Research, Vienna, Austria

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#### RESULTS AND DISCUSSION

*Porcellio scaber* ontogenetic development in the brood pouch, from released fertilized egg to marsupial manca, was described morphologically (Mlatović et al., 2010; Watt, 2009), but cuticle differentiation was not followed in detail. Here we present preliminary results of the exoskeletal cuticle ultrastructure in two stages of late embryos (Fig. 1, 2) and in marsupial mancas (Fig. 3). Late embryo epidermis is covered by disordered fibrous material, supported by a thin dense layer, altogether named embryonic cuticle (Fig. 4). The cuticle of prehatching late embryos and marsupial mancas was already differentiated in three main layers, exocuticle, endocuticle and endocuticle, and displayed architecture similar to the cuticle of adults (Fig. 5, 6). Mancae molt inside the brood pouch and here we present ultrastructural data on degraded and newly assembling cuticle in marsupial mancas (Fig. 6).

**SEM**  
Animals were stained with Azur II-Methylene I Zeiss AxioImager Z1 light microscope (SEM).

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doi: 10.3897/zookeys.55.10440  
www.zooskeys.org

RESEARCH ARTICLE



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Mrak P.<sup>1</sup>, Žnidarič N.<sup>1</sup>, Tušek-Žnidarič M.<sup>1</sup>, Gruber D.<sup>2</sup>, Klepač W.<sup>2</sup>, Šrnus J.<sup>1</sup>  
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<sup>2</sup>Department of Ultrastructural Research, University of Vienna, Vienna, Austria.

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**Methods:** A detailed study characterizes the stages of Conventional ultrastructural: performed on ultrathin section fixed specimens by energy-dispersive X-ray analysis. Results and discussion: Ultrastructural analysis was performed in vitro.

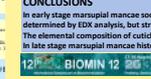
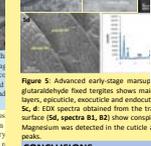
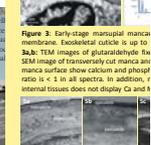
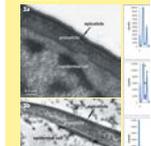
### CALCIFICATION OF CRUSTACEAN

#### INTRODUCTION

Crustacean exoskeletal cuticle is an egg or chitin-protein fibers. Mature cuticle is by calcite, amorphous calcium carbonate, and so on (Meyer 2008). The cuticle development in the female brood pouch during molting in adults. Calcification is not but data on calcification of the forming cuticle.

**AIM**  
In this study a combination of microscopic ultrastructure and composition of developmental stages of *Porcellio scaber* adult isopod crustaceans in marine pore Ultrastructure was determined by TEM embedded tissue ultrathin sections. SE method used, air dried and carbon calcified tissues screening paraffin and on.

#### RESULTS

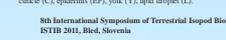
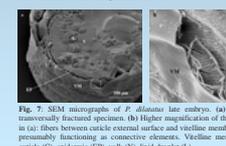
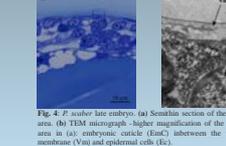
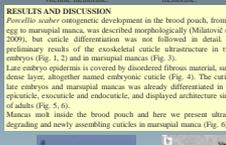


### CUTICLE STRUCTURE IN *Porcellio* EMBRYOS

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#### INTRODUCTION

Crustacean exoskeleton is a chitin-based extracellular matrix, produced by the hypodermal epithelium during embryogenesis and during molting. Cuticle synthesis implies extensive polarized secretion at the apical side of epithelial cells and sequential elaboration of cuticular layers. The knowledge on cuticle differentiation during embryonic development derives mainly from studies on the fruit fly *Drosophila melanogaster* (Mönnich, 2010). Insect and crustacean exoskeleton of these three common features regarding overall architecture and chitin-based structural framework, but on the hand differ considerably in composition. Data on terrestrial crustacean cuticle differentiation during embryonic development are very scarce. To our knowledge only one terrestrial crustacean species has been studied from this respect recently (Hlaváčková 2008). In this study we report on ultrastructural characteristics of the exoskeletal cuticle in three developmental stages within the brood pouch of isopod crustacean *Porcellio scaber* females (Fig. 1-2, 3).



**MATERIALS AND METHODS**  
Animals were maintained in a reported in Milatović et al. (2008). Embryos and marsupial manca observed by stereomicroscopy (LM) and transverse sections were stained with Azur II-Methylene Blue Ziehl-Ackermann Z11 light microscopy (SEM).

**RESULTS**  
*Porcellio scaber* ontogenetic development in the brood pouch, from released fertilized egg to marsupial manca, was described morphologically (Milatović et al., 2010; Watt 2009), but cuticle differentiation was not followed in detail. Here we present preliminary results of the exoskeletal cuticle ultrastructure in two stages of late embryos (Fig. 1, 2) and in marsupial manca (Fig. 3). Late embryo epidermis is covered by disordered fibrous material, supported by a thin dense layer, altogether named embryonic cuticle (Fig. 4). The cuticle of pre-hatching late embryos and marsupial manca was already differentiated in three main layers, exocuticle and endocuticle, and displayed architecture similar to the cuticle of adults (Fig. 5, 6). Manca molt inside the brood pouch and here we present ultrastructural data on developing and newly assembling cuticle in marsupial manca (Fig. 6).

**DISCUSSION**  
SEM images of *P. scaber* late embryos (a) Semithin section of the surface morphology - higher magnification of the squared area in (a); embryonic cuticle (EmC) between the vitelline membrane (Vm) and epidermal cells (Ec).

**DISCUSSION**  
SEM images of *P. scaber* marsupial manca (a) Semithin section of the surface morphology - higher magnification of the squared area in (a); Epidermal cell (Ec) is on assembling cuticle (NC), exocuticle (\*) and with layers: epicuticle (Ep), chitinolite (Ch), endocuticle (EnC). Cuticular scale (Sc), epidermal cell (Ec).

**DISCUSSION**  
SEM images of *P. scaber* advanced early stage marsupial manca (a) Overview of the surface morphology - higher magnification of the squared area in (a); Fibres between cuticle external surface and vitelline membrane (arrows), presumably functioning as connective elements. Vitelline membrane (VM), cuticle (C), epidermis (EP), yolk (Y), lipid droplet (L).

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## Egg envelopes and cuticle of marsupial mancas and marsupial mancas

Polona Mrak<sup>1</sup>, Nada Žnidarič<sup>1</sup>, Waltraud Klepač<sup>2</sup>, Dan

## Exoskeleton anchoring to tendon cells and muscles in molting isopod crustaceans

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### Abstract

Specialized mechanical connection between exoskeleton and underlying muscles in arthropods is a complex network of interconnected matrix constituents, junctions and associated cytoskeletal elements, which provides prominent mechanical attachment of the epidermis to the cuticle and transmits muscle tensions to the exoskeleton. This linkage involves anchoring of the complex extracellular matrix composing the cuticle to the apical membrane of tendon cells and linking of tendon cells to muscles basally. The ultrastructural architecture of these attachment complexes during molting is an important issue in relation to integument integrity maintenance in the course of cuticle replacement and in relation to movement ability. The aim of this work was to determine the ultrastructural organization of exoskeleton – muscles attachment complexes in the molting terrestrial isopod crustaceans, in the stage when integumentary epithelium is covered by both, the newly forming cuticle and the old detached cuticle. We show that the old exoskeleton is extensively mechanically connected to the underlying epithelium in the regions of muscle attachment sites by massive arrays of fibers in adult premolting *Ligia italica* and in prehatching embryos and pre-molt marsupial mancas of *Porcellio scaber*. Fibers expand from the tendon cells, traverse the new cuticle and ecdysal space and protrude into the distal layers of the detached cuticle. They likely serve as final anchoring sites before exuviation and may be involved in animal movements in this stage. Tendon cells in the prehatching embryo and in marsupial mancas display a substantial apicobasally oriented transcellular arrays of microtubules, evidently engaged in myotendinous junctions and in apical anchoring of the cuticular matrix. The structural framework of musculoskeletal linkage is basically established in described intramarsupial developmental stages, suggesting its involvement in animal motility within the marsupium.

### Keywords

cuticle, chitin, microtubules, anchoring junctions, extracellular matrix, embryo

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### Abstract

An important adaptation to land habitats in terrestrial isopods is the development of a fluid-filled female brood pouch, the marsupium. The role of egg envelopes and cuticle renewal during marsupial mancas. Egg envelopes cover embryos, their vitelline membrane throughout the whole embryo and underlying simple ultrastructural architecture compared to the cuticle of the adult. Cuticle renewal is produced in late embryonic development by hypodermal differentiation in relation to growth of developing embryos. Prehatching late-stage embryos and marsupial mancas display a thin and homogenous, the characteristic arrangement in exocuticle are hardly discernible in prehatching embryos. Differences from adult cuticle are evident in signs of cuticle renewal in prehatching embryo and hypodermis, partial disintegration of endocuticle and

### Keywords

chorion, vitelline membrane, cuticle, molting, ontogeny

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### CUTICLE STRUCTURE IN PORCELLIO EMBRYOS AND

Mrak P<sup>1</sup>, Žnidarič N<sup>1</sup>, Tušek-Žnidarič M<sup>1</sup>, Gruber D<sup>2</sup>, Klepač W<sup>2</sup>, Štrus J<sup>1</sup>  
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E-mail: polonamrak@bf.uni-lj.si, nada.znidaric@bf.uni-lj.si  
<sup>2</sup> Department of Ultrastructural Research, University of Vienna, Vienna, Austria.

**ABSTRACT:** Crustacean exoskeleton is a chitin-based extracellular matrix, produced by the epithelium during embryogenesis and during molting. Data on terrestrial crustacean cuticle embryonic development are very scarce. In this study we report on ultrastructural characteristics of cuticle development in two developmental stages within the brood pouch of *Porcellio scaber*: late embryos and marsupial mancas were already differentiated in three main layers: exocuticle and endocuticle, and displayed architecture similar to the cuticle of adults. Analyzed embryonic stages is underway to gain additional insight into crustacean cuticle embryogenesis.

**Keywords:** cuticle, embryo, isopod, ultrastructure

#### 1. Introduction

Cuticle is an apical extracellular matrix, comprising distinct horizontal layers, differing in structure and composition. It is produced by the integument, hindgut and foregut epithelia during embryonic development and it is renewed periodically during molting. Cuticle synthesis implies extensive polarized secretion at the apical side of epithelial cells and sequential elaboration of cuticular layers. The knowledge on cuticle differentiation during embryonic development derives mainly from studies on the fruit fly *Drosophila melanogaster*, as this model species offers wide opportunities for a genetic approach to address the cuticle

0.1M cacodylate buffer (pH=7.2) the vitelline membrane of emb. perforated with a thin needle. / cacodylate buffer, the samples were osmium tetroxide for 2 hours, dehydrated. Specimens for LM and TEM Agar 100 resin. Prior to embedment sections were cut with a diamond knife. Semithin sections were cut stained with Azur II-Methylene Blue. Ultrathin sections were cut with a Re ultramicrotome (Leica), contrast enhanced with 1% lead citrate and 0.5% uranyl acetate.

### CUTICLE STRUCTURE IN *Porcellio* EMBRYOS

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#### INTRODUCTION

Crustacean exoskeleton is a chitin-based extracellular matrix, produced by the hypodermal epithelium during embryogenesis and during molting. Cuticle synthesis implies extensive polarized secretion at the apical side of epithelial cells and sequential elaboration of cuticular layers. The knowledge on cuticle differentiation during embryonic development is mainly from studies on the fruit fly *Drosophila melanogaster* (Mönnich, 2010). Insect and crustacean exoskeletons of these two common features regarding overall architecture and chitin-based structural framework, but on the other hand differ considerably in composition. Data on terrestrial crustacean cuticle differentiation during embryonic development are very scarce. To our knowledge only one terrestrial crustacean has been studied from this respect recently (Silvestrov, 2008). In this study we report on ultrastructural characteristics of the exoskeletal cuticle in three developmental stages within the brood pouch of isopod crustacean *Porcellio scaber* females (Fig. 1, 2, 3).

**MATERIALS AND METHODS**  
Animals were maintained in la reported in Milatović et al. (2009) and marsupial mancas observed by stereomicroscopy (LM) and transverse sections were stained with uranyl acetate and lead citrate. *Porcellio* animals were fixed by perfusion (SEM).

**RESULTS AND DISCUSSION**  
*Porcellio scaber* ontogenetic development in the brood pouch, from released fertilized egg to marsupial mancas, was described morphologically (Milatović et al., 2010; Watt, 2009), but cuticle differentiation was not followed in detail. Here we present preliminary results of the exoskeletal cuticle ultrastructure in two stages of its development: late embryos (Fig. 1, 2) and in marsupial mancas (Fig. 3). Late embryo epidermis is covered by disordered fibrous material, supported by a thin dense layer, altogether named embryonic cuticle (Fig. 4). The cuticle of prehatching late embryos and marsupial mancas was already differentiated in three main layers, exocuticle, endocuticle and endocuticle, and displayed architecture similar to the cuticle of adults (Fig. 5, 6). Mancas molt inside the brood pouch and here we present ultrastructural data on degenerating and newly assembling cuticle in marsupial mancas (Fig. 6).

Figure 1: SEM micrographs of *P. scaber* late embryo. (a) Overview of transversely fractured specimen. (b) Higher magnification of the squared area in (a). (c) Fibers between cuticle external surface and vitelline membrane (arrows), presumably functioning as connective tissues. (d) Vitelline membrane (VM), cuticle (C), epidermis (EP), yolk (Y), lipid droplet (L).

Figure 2: SEM micrographs of *P. scaber* late embryo. (a) Overview of transversely fractured specimen. (b) Higher magnification of the squared area in (a). (c) Epidermal cell (EC) is covered by vitelline membrane (VM) and epidermal cells (EC).

Figure 3: SEM micrographs of *P. scaber* marsupial mancas. (a) Semithin surface area. (b) TEM micrograph - higher magnification of the squared area in (a). Epidermal cell (EC) is covered by vitelline membrane (VM) and epidermal cells (EC).

Further ultrastructural characterization of the cuticle in earlier embryonic stages is underway to insight into cuticle morphogenesis during embryonic development and to allow comparative analysis with other crustaceans.

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### CALCIFICATION OF CRUSTACEAN

#### INTRODUCTION

Crustacean exoskeletal cuticle is an organ on chitin-protein fibers. Mature cuticle is by calcite, amorphous calcium carbonate (ACC), and in some crustaceans, the cuticle development in the female brood pouch during molting is calcification is not but data on calcification of the forming cuticle

**AIM**  
In this study a combination of microscopic ultrastructure and composition of developmental stages of *Porcellio scaber* adult isopod crustaceans in marine pore Ultrastructure was determined by TEM embedded tissue ultrathin sections. SEM method used, air dried and carbon coated tissues scanning paraffin and on

#### RESULTS

Figure 4: SEM micrographs of *P. scaber* prehatching late embryo. (a) Semithin section of the surface membrane. Exoskeletal cuticle is up to 300 nm. TEM image of glutaraldehyde fixed SEM image of transversely cut mancas mancas surface show calcium and phosphorus is < 1 in all spectra. In addition, 4 internal tissues does not display Ca and P.

Figure 5: Early-stage marsupial mancas membrane. Exoskeletal cuticle is up to 300 nm. TEM image of glutaraldehyde fixed SEM image of transversely cut mancas mancas surface show calcium and phosphorus is < 1 in all spectra. In addition, 4 internal tissues does not display Ca and P.

Figure 6: Advanced early-stage marsupial mancas shows main features of adult crustacean cuticle. Three principal layers: exocuticle, endocuticle and endocuticle with a network of pore canals (arrows) are evident. EDS-EDX spectra obtained from the transversely fractured engine cuticle (E, spectrum A) and surface (S, spectra B, S2) show conspicuous calcium peaks, similar to the cuticle of adults. Magnesium was detected in the cuticle as well. In all spectra Ca peaks are much higher than P peaks.

#### CONCLUSIONS

Early stage marsupial mancas soon after hatching from the vitelline membrane the initial cuticle calcification is indicated as Ca, Mg and P were determined by EDX analysis, but strongly calcified tissue was not evident in histochemical staining. The elemental composition of cuticle in advanced early marsupial mancas is similar to that in adult specimens, with prominent Ca, Mg and P peaks. Late stage marsupial mancas histochemical staining, and ultrastructure reveal that exoskeleton is similar to adults.



## Egg envelopes and cuticle in marsupial and

## Exoskeleton anchoring to tendon cells and muscles

### CUTICLE STRUCTURE IN *PORCELLIO* EMBRYOS AND

Mrak P.<sup>1</sup>, Žnidarič N.<sup>1</sup>, Tušek-Žnidarič M.<sup>1</sup>, Gruber D.<sup>2</sup>, Klepal W.<sup>3</sup>, Štrus J.<sup>1</sup>  
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E-mail: polonamrak@bf.uni-lj.si, jana.strus@bf.uni-lj.si  
<sup>2</sup> Department of Ultrastructural Research, University of Vienna, Vienna, Austria.

**ABSTRACT:** Crustacean exoskeleton is a chitin-based extracellular matrix, produced by epithelium during embryogenesis and during molting. Data on terrestrial crustacean cuticle embryonic development are very scarce. In this study we report on ultrastructural characteristics of cuticle in two developmental stages within the brood pouch of *Porcellio* cuticle of late embryos and marsupial mancae was already differentiated in three main exocuticle and endocuticle, and displayed architecture similar to the cuticle of adults. Analytical embryonic stages is underway to gain additional insight into crustacean cuticle embryogenesis.

**Keywords:** cuticle, embryo, isopod, ultrastructure

**1. Introduction**  
Cuticle is an apical extracellular matrix, comprising distinct horizontal layers, differing in structure and composition. It is produced by the integument, hindgut and foregut epithelia during embryonic development and it is renewed periodically during molting. Cuticle synthesis implies extensive polarized secretion at the apical side of epithelial cells and sequential elaboration of cuticular layers. The knowledge on cuticle differentiation during embryonic development derives mainly from studies on the fruit fly *Drosophila melanogaster*, as this model species offers wide opportunities for a genetic approach to address the cuticle

0.1M cacodylate buffer (pH=7.2) the vitelline membrane of emb perforated with a thin needle. / cacodylate buffer, the samples w osmium tetroxide for 2 hours, dehydrated. Specimens for IM and TEM Agar 100 resin. Prior to embed performed with a thin needle for resin. Semithin sections were cut stained with Azur II-Methylene I Zeiss AxioImager Z1 light mi sections were cut by a Re ultramicrotome (Leica), contrast osmium and 1% lead citrate on

### Cuticle formation in *Porcellio scaber*: Im

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Keywords: embryo, marsupial ma

**Introduction:** Crustacean e cuticle during embryonic or minerals, mostly calcium car cuticle during embryonic biomineralization processes intramarsupial developmental analysis of cuticle ultrastructu of mineral component. The e environment of the female ma marsupium for another week. *Drosophila melanogaster* with and aquatic amphipod crustac

**Methods:** A detailed sta characterize the stages of Conventional ultrastructura performed on ultrathin section fixed specimens by energy-dis Results and discussion: Ul matrices was performed in tv

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In this study a combination of microscop ultrastructure and composition of developmental stages of *Porcellio scab* adult isopod crustaceans in marine pore Ultrastructure was determined by TE embedded tissue ultrathin sections. SE method used, air dried and carbon coated tissues showing paraffin and on

#### RESULTS

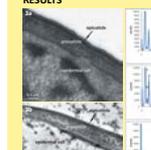


Fig. 3. P. scaber producing late embryo, vit removed. (a) Semithin section of the surface micrograph - higher magnification of the area. Cuticle differentiated in epicuticle (Ea), exo endocuticle (Ea). Cuticular scale (Sc), epidermis

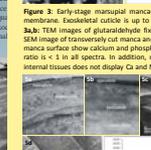


Fig. 4. P. scaber late embryo. (a) Semithin section of the surface area. (b) TEM micrograph - higher magnification of the squared area in (a); embryonic cuticle (EmC) between the vitelline membrane (Vm) and epidermal cells (Ec).

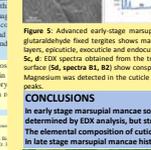


Fig. 5. P. scaber marsupial manca. (a) SEM image of transversely cut cuticle and phloft ratio is < 1. In all spectra, in addition, 4 internal tissues does not display Ca and S

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#### Abstract

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## Alizarin red S staining of the crustacean cuticle: implementation in the study of *Porcellio scaber* larvae

### Histokemijska analiza kutikule rakov z barvilom alizarin rdeče S: uporaba v proučevanju ličink raka enakonožca vrste *Porcellio scaber*

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**Abstract:** Exoskeletal cuticle of crustaceans is a chitinous matrix, produced apically by epidermis and stiffened by sclerotization and calcification. Embryos of terrestrial isopod crustacean *Porcellio scaber* develop within the female brood pouch, marsupium, and after hatching larvae mancae continue their development in the marsupium for another week. This study was performed to reveal at the histochemical level whether the exoskeletal cuticle of marsupial mancae is already calcified. Fifteen different procedures of histochemical staining with alizarin red S (ARS), established for calcified tissue localization primarily in vertebrate histology, were evaluated on mancae and adult *P. scaber* specimens. The best differential staining of the exoskeletal cuticle was obtained with neutral buffered formaldehyde fixation, followed by paraffin sections staining with ARS 1 (pH 9) or ARS 2 (pH 6.4) or ARS 3 (pH 4.8) solution. Clear differential staining was achieved also in cryosections of formaldehyde fixed samples, stained with ARS 1 solution (pH 9). Our results suggest that prominent calcification of exoskeletal cuticle is present during postembryonic development of *P. scaber* mancae in the marsupium. Exoskeleton hardening is likely important also for body movements, that we observed in mancae before they were released from marsupium. The proposed procedures of ARS method are presumed to be applicable for histochemical studies of other calcified chitinous matrices.

**Keywords:** calcification, histochemistry, larval development, terrestrial isopods, Crustacea

**Uvrlek:** Eksoskeletalna kutikula rakov je hitinski matiks na apikalni strani epidermisa. Trdnost kutikule je posledica sklerotizacije in kalcifikacije organskega matiksa. Embriji kopenskega raka enakonožca *Porcellio scaber* se razvijajo v vrečastem valniku samic, marsupiju, kjer po izleganju nadaljujejo svoj razvoj približno en teden tudi ličinke manke. S histokemijsko metodo smo ugotovili ali je

### CUTICLE STRUCTURE IN *Porcellio* EMBRYOS

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Fig. 2. P. scaber producing late embryo, vitelline membrane removed. (a) Semithin section of the surface micrograph - higher magnification of the squared area in (a); embryonic cuticle (EmC) between the vitelline membrane (Vm) and epidermal cells (Ec).

**RESULTS AND DISCUSSION**  
*Porcellio scaber* ontogenetic development in the brood pouch, from released fertilized egg to marsupial manca, was described morphologically (Mönnich et al. 2010; Watt 2009), but cuticle differentiation was not followed in detail. Here we present preliminary results of the exoskeletal cuticle ultrastructure in two stages of late embryos (Fig. 1, 2) and in marsupial mancae (Fig. 3). Late embryonic epidermis is covered by disordered fibrous material, supported by a thin dense layer, altogether named embryonic cuticle (Fig. 4). The cuticle of pre-hatching late embryos and marsupial mancae was already differentiated in three main layers, exocuticle, endocuticle and endocuticle, and displayed architecture similar to the cuticle of adults (Fig. 5, 6). Mancae molt inside the brood pouch and here we present ultrastructural data on degrading and newly assembling cuticle in marsupial mancae (Fig. 6).

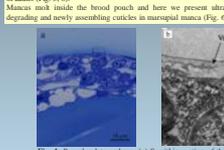


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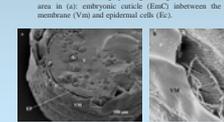


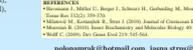
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A silhouette of a person stands on a grassy field, looking up at a vast, starry night sky. A bright, glowing blue light source is visible on the horizon, creating a vertical beam of light that illuminates the scene. The sky is filled with numerous stars of varying brightness and colors, including blue and white. The overall mood is contemplative and awe-inspiring.

**“I don't know where I'm going,**

**but I'm on my way.”**  
**~Carl Sagan**

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