UNIVERZA V LJUBLJANI
BIOTEHNIŠKA FAKULTETA

Polona MRAK

FUNKCIONALNA ULTRASTRUKTURA EPIDERMISA IN ZUNAJCELIČNIH MATRIKSOV MED RAZVOJEM KOPENSKEGA RAKA *Porcellio scaber* (CRUSTACEA: ISOPODA)

DOKTORSKA DISERTACIJA

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FUNCTIONAL ULTRASTRUCTURE OF EPIDERMIS AND EXTRACELLULAR MATRICES DURING DEVELOPMENT OF A TERRESTRIAL CRUSTACEAN *Porcellio scaber* (CRUSTACEA: ISOPODA)

DOCTORAL DISSERTATION

Ljubljana, 2015

Na podlagi Statuta Univerze v Ljubljani ter po sklepu Senata Biotehniške fakultete in sklepa seje Komisije za doktorski študij Univerze v Ljubljani z dne 19. 9. 2012 je bilo potrjeno, da kandidatka izpolnjuje pogoje za opravljanje doktorata znanosti na Interdisciplinarnem doktorskem študiju Bioznanosti, znanstveno področje: znanosti o celici. Za mentorico je bila imenovana prof. dr. Jasna Štrus. Na podlagi sklepa seje Komisije za doktorski študij Univerze v Ljubljani z dne 21. 10. 2013 je bila za mentorico imenovana doc. dr. Nada Žnidaršič in za somentorico prof. dr. Jasna Štrus.

Doktorska disertacija je rezultat raziskav, ki so potekale na Oddelku za biologijo Biotehniške fakultete Univerze v Ljubljani. Raziskave z vrstično elektronsko mikroskopijo so bile opravljene v laboratoriju za ultrastrukturne raziskave Univerze na Dunaju (Core Facility Cell Imaging and Ultrastructure Research, University of Vienna). Energijsko-disperzijska rentgenska spektrometrija je bila opravljena na Odseku za nanostrukturne materiale Inštituta Jožef Stefan v Ljubljani. Ramanska spektroskopija je bila opravljena v Laboratoriju za molekulsko fiziko Inštituta Ruđer Bošković v Zagrebu.

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Datum zagovora: 22. 4. 2015

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KLJUČNA DOKUMENTACIJSKA INFORMACIJA

ŠD Dd
DK 591:595.373:537.533.35(043.3)
KG jajčne ovojnice/hitinski matriks/prekutikularni matriks/obnavljanje kutikule/kalcifikacija/diferenciacija epitela/mišičnoskeletni sistem/embriogeneza/ličinka manka/valilnik/Isopoda/mikroskopija
AV MRAK, Polona, univerzitetna diplomirana biologinja
SA ŽNIDARŠIČ, Nada (mentorica)/ŠTRUS, Jasna (somentorica)
KZ SI-1000 Ljubljana, Jamnikarjeva 101
ZA Univerza v Ljubljani, Biotehniška fakulteta, Interdisciplinarni doktorski študij Bioznanosti, znanstveno področje znanosti o celici
LI 2015
IN FUNKCIONALNA ULTRASTRUKTURA EPIDERMISA IN ZUNAJCELIČNIH MATRIKSOV MED RAZVOJEM KOPENSKEGA RAKA *Porcellio scaber* (CRUSTACEA: ISOPODA)
TD Doktorska disertacija
OP XI, 150 str., 1 pregl., 20 sl., 3 pril., 100 vir.
IJ sl
JI sl/en
AI Eksoskeletna kutikula rakov je kalcificiran hitinski matriks, ki ga med embrionalnim razvojem in med levitvijo odraslih apikalno izloča epidermis. Embrionalni razvoj kopenskih rakov enakonožcev (Oniscidea) poteka v vodnem okolju valilnika samice. Ličinka manka se razvija v valilniku še teden dni po izleganju embrija iz vitelinske membrane. Med razvojem so embriji in ličinke obdani z različnimi matriksi, z jajčnimi ovojnicami od začetka razvoja, kasneje pa tudi z apikalnimi matriksi epidermisa. Izvedli smo ultrastrukturne analize jajčnih ovojnic, epidermalnih apikalnih matriksov in črevesnih apikalnih matriksov med razvojem mokrice *Porcellio scaber* v valilniku. Z označevanjem z lektini WGA smo na ravni elektronske mikroskopije lokalizirali makromolekule, ki vsebujejo *N*-acetilglukozamin, kar vključuje tudi hitin. Elementno in mineralno sestavo kutikule smo analizirali histokemijsko, z energijsko-disperzijsko rentgensko spektrometrijo (EDXS) ter Ramansko spektroskopijo. V primerjavi z jajčnimi ovojnicami zrelih jajčec vinske mušice, sta jajčni ovojnici embrijev mokrice tanjši, horion pa je strukturno enostavnejši. Zgodnje izločanje apikalnega matriksa epidermisa smo opazili pri srednjem embriju v stadiju 10. V nadaljnjem razvoju srednjega embrija se zaporedno izločita vsaj dva prekutikularna matriksa, ki se od eksoskeletne kutikule odraslih razlikujeta po zgradbi in sestavi. Podoben matriks je prisoten tudi v črevesu. Pri poznem embriju v stadiju 18 epidermis izloči prvi kutikularni matriks, ki se do konca embrionalnega razvoja odebeli in strukturno diferencira v troslojno kutikulo. Oblikovano je tudi strukturno ogrodje mišičnoskeletnih povezav. Ličinka manka izloči novo kutikulo, v kateri se akumulira kalcij. V nadaljnjih stadijih marzupijske manke se kutikula odebeli, kalcificira in ima podobno zgradbo in sestavo kot kutikula odraslih rakov. Črevesna kutikula še ni v tolikšni meri podobna črevesni kutikuli odraslih. Pozna marzupijska manka v fazi predlevitve tvori novo kutikulo. Strukturno organizirana, kalcificirana in z mišicami povezana kutikula marzupijskih mank ima že pomembno zaščitno in oporno vlogo in omogoča gibanje mank znotraj valilnika. Ultrastrukturna organizacija epidermalnih celic je povezana z diferenciacijo epidermisa med razvojem in s sintezo apikalnih matriksov. Opisali smo zaporedne stopnje oblikovanja medceličnih stikov in sprememb oblike celic, ki jih interpretiramo kot pokazatelje diferenciacije epidermisa. Preoblikovanje apikalne plazmaleme v nizke izbokline je povezano s sintezo in izločanjem apikalnih matriksov. Različna organiziranost Golgijevega aparata v opisanih razvojnih stadijih je verjetno povezana z diferenciacijo celic in s sintezo apikalnega matriksa.

KEY WORDS DOCUMENTATION

DN Dd
DC 591:595.373:537.533.35(043.3)
CX egg envelopes/chitinous matrix/precuticular matrix/cuticle renewal/calcification/epitelial differentiation/musculoskeletal system/embryogenesis/larva manca/marsupium/Isopoda/microscopy
AU MRAK, Polona
AA ŽNIDARŠIČ, Nada (supervisor)/ŠTRUS, Jasna (co-advisor)
PP SI-1000 Ljubljana, Jamnikarjeva 101
PB University of Ljubljana, Biotechnical faculty, Interdisciplinary Doctoral Programme in Biosciences, scientific field Cell Sciences
PY 2015
TI FUNCTIONAL ULTRASTRUCTURE OF EPIDERMIS AND EXTRACELLULAR MATRICES DURING DEVELOPMENT OF A TERRESTRIAL CRUSTACEAN *Porcellio scaber* (CRUSTACEA: ISOPODA)
DT Doctoral dissertation
NO XI, 150 p., 1 tab., 20 fig., 3 ann., 100 ref.
LA sl
AL sl/en
AB Exoskeletal crustacean cuticle is a calcified chitinous matrix, produced apically by epidermis during embryogenesis and during molting of adults. Embryonic development of terrestrial isopods (Oniscidea) takes place in aqueous environment of the female marsupium. Larva manca develops in the marsupium for a week after hatching of the embryo from the vitelline membrane. Embryos and larvae are coated by different matrices, by egg envelopes from the beginning of development and later also by apical matrices of epidermis. This study reveals ultrastructural organization of egg envelopes, epidermal apical matrices and gut apical matrices during intramarsupial development of terrestrial isopod *Porcellio scaber*. Localization of macromolecules, containing *N*-acetyl-glucosamine, including chitin, was performed by lectin WGA labelling at the level of electron microscopy. Elemental and mineral composition of the cuticle was analysed histochemically, by energy dispersive X-ray spectroscopy (EDXS) and by Raman spectroscopy. In comparison to the egg envelopes of *Drosophila* mature eggs, the egg envelopes of *Porcellio* embryos are thinner and the structure of chorion is less complex. The early deposition of the apical epidermal matrix was observed in mid-stage embryo of stage 10. In the subsequent development of mid-stage embryo, at least two successive precuticular matrices are secreted. The precuticular matrices differ from the exoskeletal cuticle of adults in structure and composition. Similar apical matrix is present in the gut. Prehatching embryo in stage 18 is the earliest developmental stage that forms cuticular matrix. Until the end of embryogenesis the cuticle thickens and structurally differentiates into three-layered cuticle. The structural framework of musculoskeletal linkage is basically established. Larva manca secretes a new cuticle, that displays calcium sequestration. In the advanced stages of manca the cuticle is thickened, prominently calcified and has a similar structure and composition as the cuticle of adult crustaceans. The gut cuticle is not structurally similar to the gut cuticle of adults. Late marsupial manca in premolt phase forms a new cuticle. Structurally elaborated, calcified cuticle, connected to the muscles in marsupial larvae already performs the functions in protection and support of the larval body and in animal mobility inside marsupium. Ultrastructural organization of epidermal cells is related to epidermis differentiation during development and to apical matrices synthesis. The progressive stages of subapical cell junctions formation and changes in cell shape were described and interpreted as the indicators of epidermis differentiation. Apical plasma membrane forms the shallow bulges, which are related to synthesis and secretion of the apical matrices. Different organization of Golgi apparatus in the represented developmental stages is probably related to cell differentiation and to the apical matrices synthesis.

KAZALO VSEBINE

KAZALO ZNANSTVENIH DEL

KAZALO PREGLEDNIC

KAZALO SLIK

KAZALO PRILOG

OKRAJŠAVE IN SIMBOLI

AAS - atomska absorpcijska spektroskopija

ACC – amorfni kalcijev karbonat

ACP – amorfni kalcijev fosfat

ARS – alizarin rdeče S

FE-SEM – vrstični elektronski mikroskop s poljsko emisijo

EDTA – etilendiamin-tetraocetna kislina

EDXS – energijsko-disperzijska rentgenska spektrometrija

EM – elektronska mikroskopija / elektronski mikroskop

ER – endoplazemski retikulum

EXAFS - rentgenska absorpcijska spektroskopija

GA – Golgijev aparat

GER – granulirani endoplazemski retikulum

GlcNAc - *N*-acetilglukozamin

SEM – vrstični elektronski mikroskop / vrstična elektronska mikroskopija

TEM – presevni elektronski mikroskop / presevna elektronska mikroskopija

WGA – angl. wheat germ agglutinin

XRD - rentgenska difrakcija

1 PREDSTAVITEV PROBLEMATIKE IN HIPOTEZE

1.1 PREDSTAVITEV PROBLEMATIKE

Zunajcelični matriksi so kompleksna omrežja polisaharidov in beljakovin, ki jih sintetizirajo in izločajo celice in so pomembna sestavina večceličnih organizmov. Številni organizmi nadzorovano tvorijo različne minerale in jih nalagajo v organski zunajcelični matriks specializiranih tkiv v procesu biomineralizacije. Najbolj razširjeni so kalcijevi minerali, ki utrjujejo matriks. Na proces biomineralizacije pomembno vplivajo tako celice, ki izločajo matriks, kot tudi organske makromolekule matriksa (Bonucci, 2007; Veis, 2008). Celice, organske molekule matriksa in minerali so torej tri komponente biomineraliziranega tkiva, ki so med seboj tesno povezane in soodvisne. Za razumevanje zgradbe, funkcije in dinamike biomineraliziranih struktur je treba poznati ultrastrukturo tkiva, celic in matriksa, organsko in mineralno sestavo matriksa ter procese tvorbe in razgradnje matriksa. Biomineralizirane strukture so morfološko in funkcijsko raznolike, najpogosteje pa služijo kot skelet za zaščito mehkih tkiv pred okoljskimi pritiski, za oporo in za pritrjanje mišic (Bonucci, 2007; Veis, 2008). V splošnem je osnovno ogrodje biomineraliziranih organskih matriksov oblikovano iz kolagenskega ali hitinskega omrežja. Kolagenski mineralizirani matriks je dobro raziskan na primerih kostnega tkiva in dentina, slabše pa so raziskani hitinski mineralizirani matriksi, ki so značilni za nevretenčarje (Bonucci, 2007).

Kutikula rakov je hitinski matriks, ki pokriva vsa ektodermalna tkiva, izpostavljena zunanjemu ali notranjemu okolju. Eksoskeletna kutikula rakov je mineraliziran matriks epidermisa in je s svojimi izrednimi mehanskimi značilnostmi, kot so trdnost in odpornost proti lomljenju in močnim silam, pomemben model za razvoj biomimetičnih materialov (Dillaman in sod., 2013). Epitelne celice v ektodermalnem delu prebavila rakov prav tako na apikalno površino izločajo kutikularni matriks, ki pa večinoma ni mineraliziran. Diferenciacija hitinskih matriksov med embrionalnim razvojem je področje intenzivnih raziskav pri žuželkah, ki imajo praviloma nemineralizirano kutikulo (Dorn, 1976; Ziese in Dorn, 2003; Konopova in Zrzavy, 2005; Moussian in sod., 2006), medtem ko je o tvorbi zgodnjih hitinskih matriksov pri rakih in o zgodnjih procesih mineralizacije bioloških matriksov v splošnem zelo malo podatkov. Predmet naše raziskave je kutikula kopenskih rakov enakonožcev ali mokric (Isopoda: Oniscidea), razširjene skupine rakov, ki so uspešno poselili kopno. Ena od prilagoditev na kopensko okolje je, da embrionalni razvoj poteka v vodnem okolju valilnika (marzupija) samice. Med razvojem so embriji obdani z različnimi zaščitnimi matriksi. Jajčne ovojnice so prisotne od začetka razvoja, zunajcelični matriksi epidermisa pa se tvorijo kasneje v razvoju. Največ podatkov o kutikuli med razvojem rakov izhaja iz študij deseteronožcev (Decapoda), škrgonožcev (Branchiopoda) in postranic

(Amphipoda). Te študije so večinoma omejene na histološke raziskave ali *in vivo* opazovanja intaktnih embrijev in ličink, ultrastrukturne raziskave teh matriksov pa so redke. Novejša ultrastrukturna študija diferenciacije kutikule pri embrijih je narejena pri vodni postranici *Parhyale hawaiensis* (Havemann in sod., 2008). Pri rakih enakonožcih oblikovanje epidermalnih matriksov med celotnim razvojem v valilniku še ni bilo natančno raziskano.

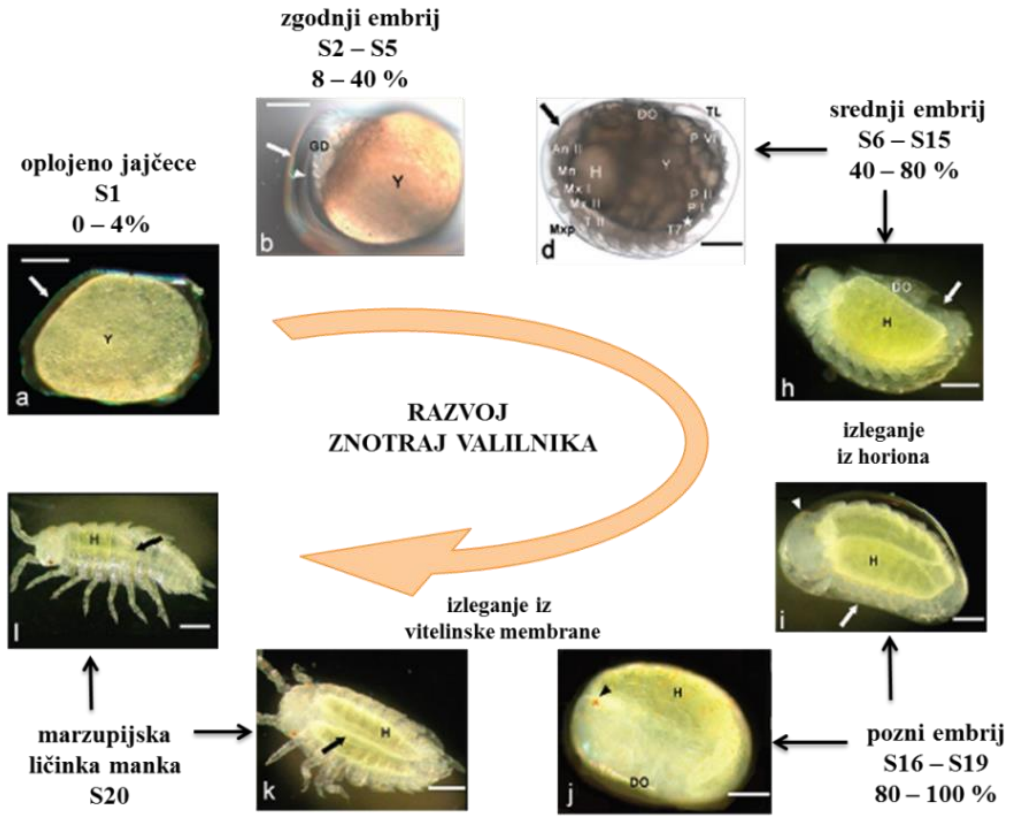
1.1.1 Zgodnji ontogenetski razvoj kopenskih rakov enakonožcev

Posebnost embrionalnega razvoja perakaridnih rakov (rakov enakonožcev, postranic in nekaj manjših skupin) je, da poteka v valilniku (marzupiju). Valilnik je s tekočino napolnjena vrečasta struktura na trebušni strani telesa samice. Nastane pred sprostitvijo oocitov iz ovidukta, s parturielno levitvijo samic. Gradi ga pet parov lističastih oostegitov, ki izraščajo iz bazalnih členov sprednjih pereopodov. Pari oostegitov se mediano prekrivajo in tvorijo ventralno steno valilnika, ki je dorzalno omejen s sterniti. Valilnik kopenskim enakonožcem omogoča razmnoževanje na kopnem, saj zagotavlja vodno okolje, potrebno za razvoj zarodkov do prostoživeče kopenske ličinke (Hoese in Janssen, 1989; Surbida in Wright, 2001; Hornung, 2011; Warburg, 2011). Z marzupijsko tekočino zagotavlja zarodkom tudi mehansko zaščito, oskrbo s kisikom, ioni in hranili ter zaščito pred bakterijskimi okužbami. Marzupijsko tekočino izloča materino telo, vzdržujejo jo kotiledoni. Voda in ioni izhajajo iz hemolimfe matere in se izločijo v valilnik skozi epitel kotiledonov, ki izraščajo iz sternitov (Hoese in Janssen, 1989). Pri kopenskih rakih enakonožcih (Oniscidea) sta opisana dva različna tipa valilnika, amfibijski ali odprti tip in kopenski ali zaprti tip (Hoese in Janssen, 1989). Valilnik amfibijskega tipa je delno odprt, kar omogoča pritok vode iz zunanjosti v marzupijsko tekočino. To je prvotni tip, ki se je ohranil pri amfibijski družini Ligiidae. Valilnik zaprtega tipa je bolj izoliran in omogoča, da je zgodnji razvoj neodvisen od zunanjih vodnih virov in tako predstavlja ključno prilagoditev kopenskih rakov enakonožcev na kopensko okolje. Ionsko sestavo in osmotsko ravnovesje marzupijske tekočine med razvojem vzdržuje transportni epitel kotiledonov (Surbida in Wright, 2001).

Slika 1: Samica *P. scaber* z valilnikom na trebušni strani telesa.
Figure 1: Female of *P. scaber* with marsupium on the ventral side of the body.

Predstavnik podreda Oniscidea, navadni prašiček *Porcellio scaber* Latreille, 1804, je ustrezen modelni organizem za študije razvoja in prilagoditve kopenskemu načinu življenja. Njegov ontogenetski razvoj od oplojenega jajčeca do marzupijske ličinke manke poteka v valilniku zaprtega tipa (sl. 1). Razvoj v valilniku traja približno 35 dni v laboratorijskih razmerah in je natančno opisan v dvajsetih morfološko različnih zaporednih razvojnih stadijih (sl. 2) (Wolff, 2009; Milatovič in sod., 2010). Jajčna celica raka *P. scaber* je makrolecitalna in centrolecitalna, torej vsebuje veliko centralno nameščenega rumenjaka. Obdajata jo dve jajčni ovojnici: vitelinska membrana, ki leži tesno ob površini in horion, ki je odmaknjen od površine jajčeca. Embrionalni razvoj lahko v grobem razdelimo na obdobje zgodnjega embrija, srednjega embrija in poznega embrija (Milatovič in sod., 2010). V obdobju zgodnjega embrija potekata brazdanje in gastrulacija. Vidna je velika masa rumenjaka in embrionalno tkivo na delu površine embrija, zasnov za okončine pa še ni. Brazdanje sprva poteka kot delitev jeder znotraj rumenjaka, po pomiku jeder na površino pa je brazdanje površinsko in nastane zarodni disk. Ko se jedra nakopičijo v predelu zarodnega diska, se začnejo sintetizirati celične membrane, ki ločijo jedra v celične strukture (celularizacija). Gastrulacija se začne kot ingresija, potovanje posameznih celic skozi gastrulacijski center, ki je nameščen približno v sredini zarodne plošče. S potovanjem celic v notranjost embrija nastane ektoderm na površini, v notranjosti pa mezendodermalna celična masa, ki se kasneje predeli v mezoderm in endoderm. Zarodni disk se podaljša v antero-posteriorni osi embrija in nastane zarodni pas, ki se v nadaljevanju diferencira v telesne člene ali somite. Embrij preide v obdobje srednjega embrija, ko se pojavijo zasnove za okončine. Razvoj okončin poteka v antero-posteriorni smeri. V tem času se iz ektoderma uvihata in podaljšujeta sprednje in zadnje črevo (stomodeum in proktodeum). Iz endoderma nastaneta zasnovi dveh cevk prebavnih žlez, hepatopankreasa, v katere se postopoma vključuje rumenjak. Srednji embrij je dorzalno upognjen, prepoznamo ga po značilni

obliki vejice ali črke "C" (Milatovič in sod., 2010). Natančnejši stadij srednjega embrija lahko določimo po naraščajočem deležu rumenjaka v prebavnih žlezah, ki se podaljšujejo proti zadnjemu delu embrija. Okončine postanejo členjene. Ob koncu obdobja srednjega embrija in začetku obdobja poznega embrija je rumenjak v celoti vključen v prebavne žleze, razvijati pa se začneta drugi cevki prebavnih žlez. Sprednje in zadnje črevo se povežeta v enotno prebavno cev. Prebavna cev je namreč pri povsem kopenskih oniscidih v celoti ektodermalnega izvora, srednje črevo pa se ohrani le kot parne cevke prebavnih žlez hepatopankreasa (Štrus in sod., 1995). Embrij se v času prehoda iz srednje v pozno razvojno obdobje izleže iz horiona in v dorzoventralni osi zavrti znotraj vitelinske membrane (Milatovič in sod., 2010). Pozni embrij je ventralno ukrivljen, vidna je rahla pigmentiranost integumenta in dobro diferencirana glava s parom anten in s pigmentiranimi očmi. V času pozne embriogeneze embrij raste znotraj vitelinske membrane. V prebavilu se posamezni predeli diferencirajo in tvorijo specializirane strukture, kot so želodčni filtri, dorzalna guba zadnjega črevesa (tiflosolis) in kutikularni matriks, ki prekriva svetlinsko površino črevesa (Štrus in sod., 2008). Na koncu embrionalnega razvoja se embrij izleže iz vitelinske membrane in postane ličinka, ki je po telesni zgradbi podobna odrasli živali. Prebavilo je morfološko diferencirano in funkcionalno za transportno in prebavno vlogo. Do sprostitve iz valilnika ličinko imenujemo marzupijska manka. Postembrionalni razvoj v valilniku traja do deset dni (Milatovič in sod., 2010). V tem času se količina rumenjaka v prebavnih žlezah zmanjšuje zaradi intenzivne porabe, kromatofore v integumentu pa so vedno številčnejše. Manka, ki se sprosti iz valilnika, ima šest parov pereopodov (Wolff, 2009; Milatovič in sod., 2010), sedmi par pereopodov pa se razvije med razvojem postmarzupijske manke (Tomescu in Craciun, 1987).



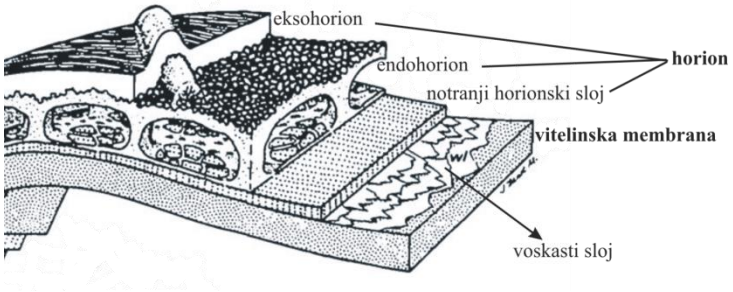
Slika 2: Obdobja razvoja raka enakonožca *P. scaber* v valilniku, od oplojenega jajčeca, zgodnjega, srednjega in poznega embrija, do marzupijske manke (prirejeno po Milatovič in sod., 2010). Pri embrionalnih obdobjih so podani razvojni stadiji (S) in časovni odstotki razvoja. Oplojeno jajčece je napolnjeno z zrnci rumenjaka (Y), na površini pa ni vidnih celic; ⇨ – horion (slika a). Pri zgodnjem embriju je na površini vidna zarodna plošča (GD); ⇨ – horion; ▷ vitelinska membrana, dvignjena nad zarodno ploščo (slika b). Pri srednjem embriju se začne razvoj okončin (An-antene, Mn-mandibule, Mx-maksile, T-torakopodi, P-pleopodi) in postopno vključevanje rumenjaka v zasnove prebavnih žlez, hepatopankreasa (H); Tl – telzon; DO – dorzalni organ (sliki d in h). Pozni embrij je obdan le z vitelinsko membrano in je ventralno ukrivljen. Telesna stena je rahlo pigmentirana, na glavi pa so vidne pigmentirane oči (▷) (sliki i in j). Ličinka manka (marzupijska manka) po izleganju iz vitelinske membrane še nekaj časa raste in se razvija v valilniku, količina rumenjaka v prebavnih cevkah hepatopankreasa (H) pa se zaradi porabe postopoma zmanjšuje (➡) (sliki k in l). Merila: 200 µm.

Figure 2: Periods of *P. scaber* intramarsupial development, from fertilized egg, early-, mid- and late-stage embryos, to marsupial manca (adapted from Milatovič et al., 2010). Developmental stages (S) and temporal procentages of development are specified in embryonic periods. Fertilized egg is filled with small yolk granules (Y) and no cells are visible on the surface; ⇨ – chorion (image a). Germ disc (GD) is visible on the surface of early- stage embryo; ⇨ – chorion; ▷ vitelline membrane, detached above the germ disc (image b). In mid-stage embryo developing limb buds (An-antennae, Mn-mandible, Mx-maxillae, T-thoracopods, P-pleopods) and yolk gradually enclosing in hepatopancreatic glands primordia (H) are visible; Tl – telson; DO – dorsal organ (images d and h). Late-stage embryo is ventrally bent and enveloped by the vitelline membrane only. The body wall is slightly pigmented and on the head pigmented eyes are notable (▷) (images i and j). Larva manca (marsupial manca) grows and develops in marsupium for a few days. Due to consumption the yolk inside the hepatopancreas tubes (H) is reduced during manca development (➡) (images k and l). Bars: 200 µm.

1.1.2 Zgradba jajčnih ovojnic členonožcev

Jajčne ovojnice so specializirani matriksi, ki obdajajo in ščitijo jajčne celice med zorenjem (oogenezo) in so prisotne tudi med embrionalnim razvojem. Te ovojnice ščitijo predvsem pred izsušitvijo in osmotskim stresom. Hkrati morajo omogočiti izmenjavo plinov med embrijem in okoljem. Pri členonožcih poznamo dve ovojnici, zunanji horion in notranjo vitelinsko membrano. Za vinsko mušico je znano, da jajčne ovojnice izločajo folikularne celice ovarijskega folikla, v katerem zori jajčna celica (Waring, 2000). Različna zgradba in kompleksnost jajčnih ovojnic nevretenčarjev je odraz embrionalnega razvoja, ki poteka v različnih okoljih. V splošnem je razvoj debelejših zaščitnih jajčnih ovojnic ali izvenembrionalnih membran povezan z življenjem v sladkih vodah in na kopnem, kjer je velik osmotski stres. O ultrastrukturi (Margaritis in sod., 1980), morfogenezi (Waring, 2000) in molekulski sestavi (Waring, 2000; Jagadeeshan in Singh, 2007) jajčnih celic je precej znanega iz proučevanj jajčnih celic modelnega nevretenčarskega organizma vinske mušice *Drosophila melanogaster*. Embrije rakov v splošnem osmotsko ščitijo jajčne ovojnice ali valilnik samice, razviti pa so lahko tudi začasni ali trajni osmoregulacijski organi, kot je dorzalni organ (Charmantier in Charmantier-Daures, 2001).

Pri vinski mušici je horion precej kompleksno zgrajen in diferenciran v tri sloje (sl. 3). Zunanji sloj, eksohorion, je vlaknaste strukture. Pod njim je kompleksen proteinski endohorion, ki je perforiran. Prostori v endohorionu naj bi imeli vlogo pri respiraciji, saj pri odloženih jajčecih olajšujejo izmenjavo plinov (Margaritis in sod., 1980; Waring, 2000). Najbolj tanek sloj horiona je notranji horionski sloj, ki leži nad vitelinsko membrano iz morfološko homogenega proteinskega matriksa. Med horionom in vitelinsko membrano je tanek voskasti sloj, ki med oogenezo nastane s kopičenjem veziklov, napolnjenih z lipidi, in naj bi pri embrijih preprečeval izgubo vode (Waring, 2000). Poleg tega ima površina jajčnega ovoja pri vinski mušici tudi specialne strukture. Mikropila je ozka struktura, ki je izbočena iz površine jajčeca in jo gradita obe ovojnici. Omogoča dostop spermija do celične membrane oocita in hkrati zaradi svoje ozkosti preprečuje polispermijo. Očitni so tudi dolgi respiracijski filamenti, polni zračnih prostorov, ki sodelujejo pri respiraciji (Waring, 2000).

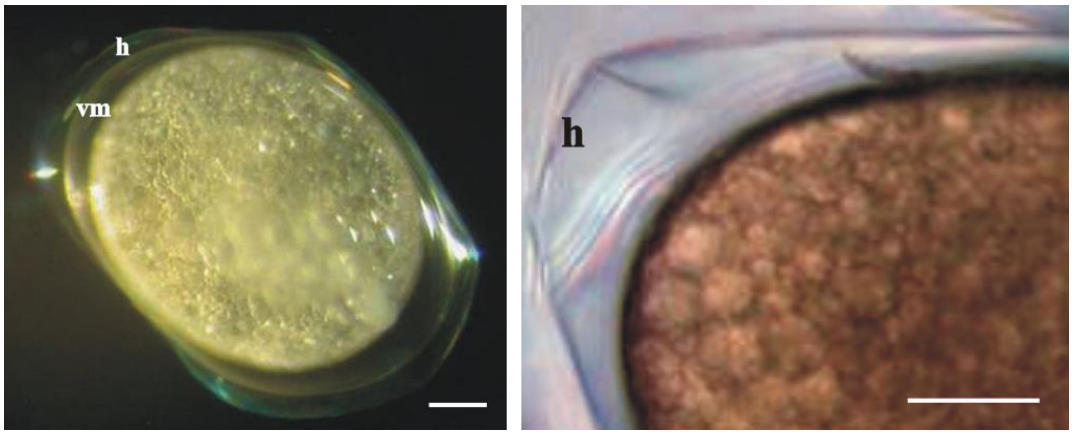


Slika 3: Shema strukture jajčnih ovojnic pri jajčni celici vinske mušice *Drosophila melanogaster* (prirejeno po Margaritis in sod., 1980: 4). Kompleksno zgrajen horion ima tri sloje: površinski eksohorion, perforiran endohorion in tanek notranji horionski sloj. Vitelinska membrana je notranja ovojnica, ki jo gradi morfološko homogen matriks. Med njo in horionom leži tanek voskasti sloj.

Figure 3: Schematic representation of the *Drosophila* egg envelopes (eggshell) (adapted from Margaritis et al., 1980: 4). Chorion has a complex structure with three layers: exochorion on the surface, endochorion with cavities and thin innermost chorionic layer. The vitelline membrane is the inner envelope, composed of morphologically homogenous matrix. A thin wax layer lies between the vitelline membrane and the chorion.

Kljub temu, da obe skupini členonožcev, žuželke in kopenski raki enakonožci, živijo na kopnem, poteka embrionalni razvoj v različnih razmerah. Embrionalni razvoj vinske mušice poteka neodvisno od samice, medtem ko se embriji rakov enakonožcev razvijajo v valilniku samice. Zato so embriji vinske mušice neposredno izpostavljeni zunanjim vplivom okolja (suši, deževju itd.), kar pomeni, da so jajčne ovojnice edina pregrada med embrijem in okoljem. Pri rakih enakonožcih je osmotska in ionska regulacija embrijskega mikrookolja delno zagotovljena z regulacijo sestave marzupijske tekočine (Surbida in Wright, 2001). Študije kažejo, da homeostatsko kontrolo embrijskega mikrookolja dodatno zagotavljajo jajčne ovojnice in dorzalni organ embrija (Surbida in Wright, 2001; Wright in O'Donnell, 2010). K visoki toleranci zgodnjih embrijev na osmotski stres naj bi prispeval predvsem horion, slabo prepusten za vodo in raztopine (Surbida in Wright, 2001). V kasnejših stadijih embrionalnega razvoja vlogo aktivnega transporta ionov in s tem vlogo v fiziološki kontroli embrijskega okolja pripisujejo predvsem dorzalnemu organu (Meschenmoser, 1989; Wright in O'Donnell, 2010). Dorzalni organ je sedlast organ pod vitelinsko membrano na dorzalni strani embrija, kjer je direktna povezava embrija z ovojnico. Podrobna ultrastruktura dorzalnega organa je opisana pri postranici *Orchestia cavimana* (Meschenmoser, 1989). O ultrastrukturi jajčnih ovojnic kopenskih rakov enakonožcev ni podatkov. Tako kot pri vseh členonožcih, jajčno celico in zgodnje embrionalne stadije raka enakonožca rodu *Porcellio* pokrivata horion in vitelinska membrana (sl. 4). Embriji se iz horiona izležejo pri prehodu v obdobje poznega embrija, ko se spremeni tudi zunanja oblika embrija,

vitelinska membrana pa obdaja embrij do izleganja v marzupijsko ličinko manko (Milatovič, 2010).



Slika 4: Oplojena jajčna celica iz valilnika raka enakonožca *P. scaber*. Leva slika: Vidni sta dve jajčni ovojnici, ki obdajata jajčece, horion (h) in vitelinska membrana (vm) (Štrus in sod., 2008). Desna slika: Viden je horion (h), ki ohlapno obdaja jajčece. Vitelinska membrana se tesno prilega površini jajčeca in na sliki ni opazna (prirejeno po Wolff, 2009). Merilo: 100 µm.

Figure 4: Fertilized egg, isolated from the marsupium of *P. scaber*. Left image: Two egg envelopes are visible around egg, chorion (h) and vitelline membrane (vm) (Štrus et al., 2008). Right image: The chorion (Ch) loosely encloses the egg. The vitelline membrane is not visible in the image as it tightly encloses the egg (adapted from Wolff, 2009). Bars: 100 µm.

1.1.3 Zgradba in obnavljanje eksoskeletne kutikule členonožcev

1.1.3.1 Struktura, sestava in obnavljanje eksoskeletne kutikule rakov in žuželk

Kutikula členonožcev je kompleksen, hierarhično urejen zunajcelični matriks, ki ga apikalno izločajo krovni epiteli (Neville, 1984). Pokriva in ščiti vsa ektodermalna tkiva, ki so izpostavljena zunanjemu ali notranjemu okolju. Sestavljajo jo hitinsko-proteinska vlakna, različni proteini in lipidi. Eksoskeletno kutikulo izloča enoslojni epidermis na površini telesa. Organizirana je v tri glavne vodoravne sloje: epikutikula, eksokutikula in endokutikula, ki se razlikujejo v strukturi, funkciji in molekulski sestavi (Roer in Dillaman, 1984; Dillaman in sod., 2013; Chapman in Merzendorfer, 2013; Moussian, 2013). Epikutikula je tanek površinski sloj iz lipidov in proteinov, deluje kot difuzijska pregrada in oblikuje različne površinske strukture, kot so kutikularne luske in grebeni. Epikutikula je sestavljena iz zunanje epikutikule, pri žuželkah imenovane tudi ovoj ('envelope'), z izmenjajočimi elektronsko gostimi in elektronsko svetlimi podsloji, in debelejše notranje epikutikule, ki vsebuje amorfni material in navpična epikutikularna vlakna. Prokutikula je notranji hitinsko-proteinski matriks. Ločimo eksokutikulo in bazalno endokutikulo, ki se s tehniko presevne elektronske mikroskopije razlikujeta po

ultrastrukturi in kontrastu podslojev ali lamel. Lastnosti kutikule rakov, kot sta mehanska trdnost in čvrstost, sta posledica kompleksne hierarhične organiziranosti hitinsko-proteinskih vlaken, ki je od molekulskega do tkivnega nivoja natančno raziskana pri rakih deseteronožcih (Decapoda) (Raabe in sod., 2006; Romano in sod., 2007; Fabritius in sod., 2009; Dillaman in sod., 2013). Hitin je biopolimer, linearni polisaharid iz *N*-acetilglukozaminov (GlcNAc), povezanih z β-1,4 glikozidnimi vezmi, antiparalelno urejene hitinske verige pa sestavljajo hitinska vlakna. Skupine hitinskih vlaken so povezane s proteini v hitinsko-proteinska vlakna, ki so razporejena v plasteh, ki potekajo vzporedno z apikalno površino epitelnih celic. Plasti hitinsko-proteinskih vlaken so naložene ena na drugo in vsaka plast je v osi poteka vlaken rahlo zasukana glede na prejšnjo. Vsak podsloj ali lamela v eksokutikuli ali endokutikuli je iz helikoidalno urejenih plasti hitinsko-proteinskih vlaken, ki so med sabo zasukane za 180°. Okrog pornih kanalov, ki potekajo navpično skozi kutikulo, so vlakna upognjena (Romano in sod., 2007; Dillaman in sod., 2013). Takšna organizacija omogoča raznolike funkcije kutikule, predvsem mehansko oporo telesu, gibljivost v povezavi z mišicami, zaščito pred dehidracijo, predatorji in okužbami ter komunikacijo z okoljem.

Trdnost kutikule je pri vseh členonožcih posledica sklerotizacije organskega matriksa (medsebojnih povezav kutikularnih proteinov), eksoskeletna kutikula pri rakih pa je poleg tega še kalcificirana. Mineralna komponenta je v popolnoma kalcificirani kutikuli odraslih rakov vključena v organski matriks ekso- in endokutikule in vsebuje kalcijev karbonat v kristalni obliki (kalcit in magnezijev kalcit), amorfni kalcijev karbonat (ACC) in amorfni kalcijev fosfat (ACP) (Roer in Dillaman, 1984; Becker in sod., 2005; Dillaman in sod., 2005; Hild in sod., 2008, 2009; Luquet, 2012). Kristalni mineral je razporejen vzdolž hitinsko-proteinskih vlaken v obliki sferičnih delcev (Roer in Dillaman, 1984; Romano in sod., 2007). Amorfna oblika kalcijevega karbonata v kutikuli rakov je stabilna, vlogo pri stabilizaciji pa pripisujejo različnim komponentam kutikule: makromolekulam, magnezijevim ionom in fosfatnim ionom (Al-Sawalmih in sod., 2009; Luquet, 2012). Prisotnost stabilnih amorfnih oblik mineralov v kutikuli prispeva k fleksibilnosti in plastičnosti in hkrati zagotavlja optimalno čvrstost in trdnost biomateriala (Al-Sawalmih in sod., 2009; Luquet, 2012). Pomembna vloga amorfnega kalcijevega karbonata je zaradi večje topnosti v primerjavi s kalcitom tudi lažja mobilizacija kalcija v fazi predlevitve pri delni dekalcifikaciji stare kutikule (Neues in sod., 2011; Luquet, 2012).

Kutikula rakov je zaradi ciklične tvorbe in razgradnje organsko-mineralnega matriksa med levitvijo pomemben model za proučevanje dinamike biomineraliziranih matriksov. Raki kutikulo obnavljajo periodično vse življenje v povezavi z rastjo in razmnoževanjem. Morfološko spreminjanje kutikule in epidermalnih celic med levitvenim ciklom je natančno opisano pri različnih vrstah rakov (Roer in Dillaman, 1984; Compere in Goffinet, 1987a, 1987b; Dillaman, 2005), vključno z mokricami

(Price in Holdich, 1980; Compere, 1991; Štrus in Compere, 1996; Ziegler, 1997; Glötzner in Ziegler, 2000; Štrus in Blejec, 2001; Neues in sod., 2011; Vittori in sod, 2012). Med levitvijo žival odvrže stari eksoskelet in ga nadomesti z novim. Levitveni cikel se začne s fazo predlevitve (angl. premolt), ko se stara kutikula začne razgrajevati in se odmakne od epitela (apoliza) ter se hkrati tvorijo predlevitveni sloji nove kutikule (epi- in eksokutikula) na apikalni površini epitelnih celic. V tem času poteka časovna in prostorska koordinacija procesov sinteze, izločanja in ureditve komponent nove kutikule v hierarhično strukturo ter razgradnje in resorpcije komponent stare kutikule. Staro in novo kutikulo ločuje kompleksen levitveni prostor. V fazi levitve (angl. ecdysis) se stara kutikula odvrže, sinteza nove kutikule pa se nadaljuje. V fazi po levitvi (angl. postmolt) se kutikula dokončno oblikuje in mineralizira, sledi faza med levitvama (angl. intermolt). Natančnejše raziskave kalcifikacije kutikule v obdobju po levitvi so pokazale, da je silicij v amorfni hidratirani obliki (SiO_2 x nH_2O) pomembna komponenta matriksa v zgodnji kalcifikaciji (Matsko in sod., 2011) in da se nalaganje kalcija začne v predelu med epi- in eksokutikulo in znotraj eksokutikule (Dillaman in sod., 2005). Znano je tudi, da je v kutikuli najprej prisoten amorfni kalcijev karbonat, šele kasneje pa tudi kalcit (Dillaman in sod., 2005; Neues in sod., 2011).

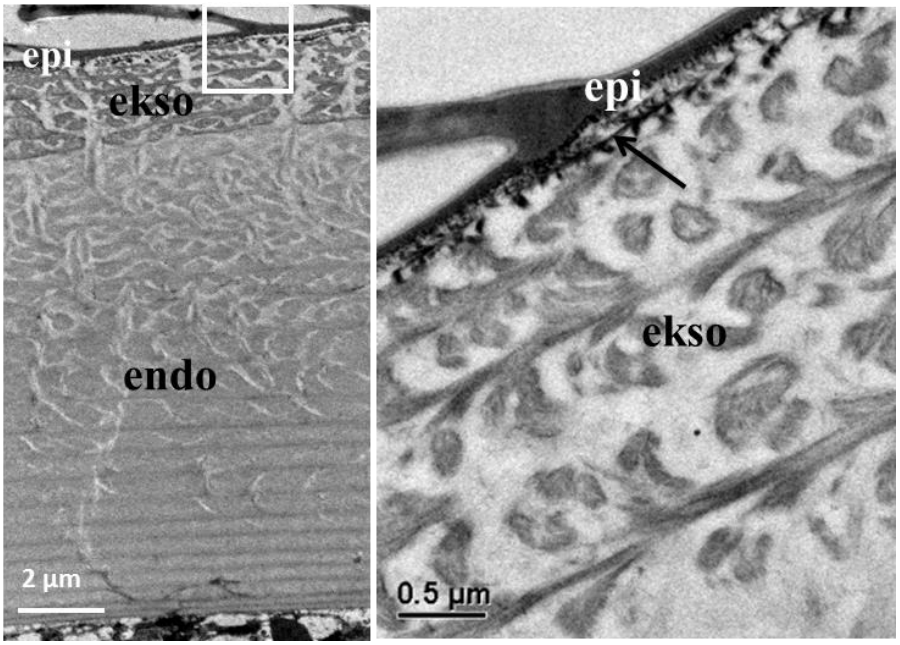
1.1.3.2 Zgradba in obnavljanje eksoskeletne kutikule kopenskih rakov enakonožcev (Isopoda)

Struktura, sestava in delovanje kutikule mokric so odraz prilagoditev na različne kopenske habitate. Ultrastruktura kutikule odraslih mokric je dobro raziskana pri nekaterih vrstah (Price in Holdich, 1980; Compere, 1991; Štrus in Compere, 1996; Ziegler, 1997; Štrus in Blejec, 2001; Hild in sod., 2009; Seidl in sod., 2011; Seidl in Ziegler, 2012, Vittori in Štrus, 2014). V tanki epikutikuli razločimo elektronsko svetlejšo zunanjo epikutikulo in elektronsko gosto notranjo epikutikulo. Zunanja epikutikula ni homogena, ampak vsebuje podsloje različnih elektronskih gostot. Za notranjo epikutikulo so značilni epikutikularni kanalčki, ki potekajo pravokotno na površino. V eksokutikuli je jasno razviden značilen vzorec razporeditve hitinsko-proteinskih vlaken, ki daje videz razvejanih snopov (sl. 5). V nekaterih študijah je opisan tudi poseben distalni predel eksokutikule z izrazito elektronsko gostimi vlakni (Hild in sod., 2009; Seidl in Ziegler, 2012). Lamelarna endokutikula vsebuje številne helikoidalno razporejene podsloje hitinsko-proteinskih vlaken. Najbolj notranji sloji kutikule so tanjši, homogeni in elektronsko gostejši, kar nekateri avtorji imenujejo membranski sloj (sl. 5).

Nedavne raziskave pri odraslih mokricah so pokazale specifičnost razporeditve različnih oblik kalcijevega karbonata po posameznih kutikularnih slojih (Hild in sod., 2008, 2009; Neues in sod., 2011; Seidl in sod., 2011). Kalcit je omejen na eksokutikulo,

medtem ko je v endokutikuli le amorfni mineral. Razporeditev bolj topnih amorfnih mineralov v proksimalnih delih kutikule povezujejo z olajšano resorpcijo kalcijevih mineralov v fazi predlevitve.

Posebnost mokric je dvodelna levitev, časovni zamik v levitvi zadnjega in sprednjega dela telesa, kar je prilagoditev na kopenske habitate. Večina mokric ima v fazi predlevitve sternalne depozite na štirih sprednjih sternitih, kjer se v levitvenem prostoru nalaga kalcij in se po levitvi porablja za mineralizacijo nove kutikule. Faza med levitvama obeh polovic se imenuje medlevitev (angl. intramolt), ko je sprednji del telesa (do vključno 4. telesnega člena) v fazi predlevitve, zadnji del pa v fazi po levitvi.



Slika 5: Ultrastruktura kutikule odrasle živali raka enakonožca *P. scaber* (arhiv Skupine za funkcionalno morfološke in ekotoksikološke raziskave nevretenčarjev, Oddelek za biologijo, Biotehniška fakulteta, Univerza v Ljubljani). Leva slika prikazuje vse sloje kutikule: epikutikula (epi), eksokutikula (ekso) in endokutikula (endo), kot si sledijo od zunanjega (distalnega) proti notranjemu (proksimalnemu) delu. Endokutikula vsebuje lamelarne podsloje hitinsko – proteinskih vlaken. Desna slika prikazuje zgornji predel kutikule (bel kvadrat na levi sliki) na večji povečavi. Epikutikula (epi) je tanek, elektronsko gost sloj, iz katerega izraščajo kutikularne luske. Eksokutikula (ekso) vsebuje hitinsko - proteinska vlakna, ki so urejena v značilen vzorec. Vlakna v distalnem predelu eksokutikule so izrazito elektronsko gosta (→).

Figure 5: Ultrastructure of the exoskeletal cuticle in adult *P. scaber* (images from Research group for functional morphology and ecotoxicology of invertebrates, Department of biology, Biotechnical faculty, University of Ljubljana). Left image shows cuticle, organized in distinct horizontal layers, arranged from outer (distal) to inner (proximal) cuticle portion: epicuticle (epi), exocuticle (ekso) and endocuticle (endo). Endocuticle comprises lamellar chitin-protein sublayers. Right image is a higher magnification of cuticle outer region (white square in the left image). Epicuticle (epi) is a thin and electron dense layer with prominent scales. Exocuticle (ekso) comprises chitin–protein fibers, arranged in a characteristic pattern. The fibers in the distal portion of the exocuticle are intensely electron dense (→).

1.1.4 Tvorba kutikule pri členonožcih

1.1.4.1 Ultrastruktura epitelnih celic in sinteza kutikule

Kutikula se tvori in diferencira med embriogenezo in pri obnavljanju med levitvijo. Med tvorbo matriksa poteka sinteza in izločanje kutikularnih komponent in spremembe oblike, velikosti in ultrastrukture epitelnih celic. Morfološko poznavanje sinteze kutikule izhaja predvsem iz študij odraslih rakov med levitvijo (poglavje 1.3.1) in ličink žuželk med levitvijo (Locke, 1961, 2001; Locke in Huie, 1979), nekaj pa je tudi morfoloških študij diferenciacije kutikule med embriogenezo žuželk (Ziese in Dorn, 2003; Konopova in Zrzavy, 2005; Moussian in sod., 2006). Celični mehanizmi diferenciacije kutikule so genetsko in molekulsko raziskani predvsem pri različnih vrstah žuželk med levitvijo ličink, med embriogenezo pa večinoma pri vinski mušici *Drosophila melanogaster*, kar je povzeto v Moussian (2010) in Moussian (2013).

Polarna organizacija epitelnih celic, ki izločajo kutikulo, je predpogoj za pravilno odlaganje kutikule na apikalni površini (Payre, 2004; Moussian, 2013). Na bazalni površini se celice povezujejo z bazalno lamino. Lateralna plazmalema povezuje sosednje celice, specializirani predeli lateralne plazmaleme pa so diferencirani v medcelične stike, ki imajo vlogo mehanskih povezav in komunikacije med celicami. Adherentni stiki so nameščeni najbolj apikalno in se znotraj celice povezujejo z aktinskim citoskeletom, kar pripomore k stabilizaciji tkiva. Pod njimi so septirani (pregradni) stiki, ki z zaprtjem medceličnega prostora delujejo kot pregrada za paracelularni transport snovi. Za medcelično komunikacijo so pomembni presledkovni stiki, ki so prisotni na lateralni plazmalemi.

Komponente kutikule ali njihovi prekurzorji se začnejo sintetizirati v citosolu in organelih polariziranih epitelnih celic, in se nato izločijo skozi apikalno plazmalemo v zunajcelični prostor. V zunajceličnem prostoru se makromolekule uredijo v hierarhično strukturo kutikule, za kar je potrebna tudi aktivnost določenih zunajceličnih encimov (sl. 6) (Moussian, 2013). Apikalna plazmalema aktivno sodeluje pri tvorbi kutikule. Tako pri ličinkah žuželk kot pri odraslih rakih v levitvi je apikalna plazmalema epitelnih celic diferencirana v strukture, ki jim avtorji pripisujejo vlogo pri sintezi in izločanju kutikularnih komponent. Prisotne so membranske izbokline ali mikrovilom podobne strukture, ki so različnih velikosti in imajo elektronsko goste konice (Locke, 1961; Locke in Huie, 1979; Koulish in Klepal, 1981; Compere, 1995; Ziegler, 1997; Elliot in Dillaman, 1999; Locke, 2001; Vittori in sod., 2012). Elektronsko goste konice izboklin se imenujejo plaki plazmaleme, za katere se predvideva, da so mesta agregacij encimov, kjer poteka sinteza hitina. V skladu s tem je pri žuželkah imunohistokemijsko potrjena lokalizacija encima hitin sintaze v skrajnem apikalnem delu epidermisa in na apikalnih

konicah mikrovilov v epitelu srednjega črevesa (Zimoch in Merzendorfer, 2002; Merzendorfer in Zimoch, 2003).

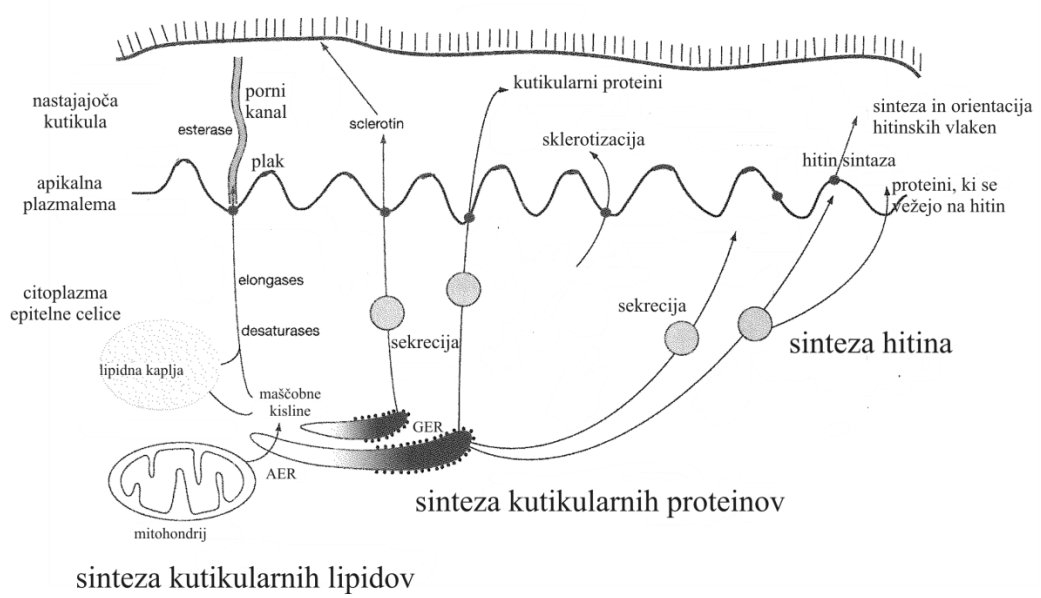
Največ novejših raziskav o biosintetski poti hitina s ključnim encimom hitin sintazo izhaja iz študij gliv in žuželk (pregledno v Merzendorfer, 2006, 2011). Nekaj podatkov o sintezi hitina in encima hitin sintaze pri rakih je v starejših študijah škrgonožca rodu *Artemia*, kjer je sinteza hitina v splošnem podobna kot pri žuželkah (Horst in sod., 1993). Vir monomerov, ki nastanejo v citoplazmi, je glukoza oziroma njena zaloga v obliki glikogena ali trehaloze. Encim hitin sintaza, ki je vgrajen v celično membrano, povezuje monomere *N*-acetilglukozamina (GlcNAc) na citoplazemski strani, nastajajoči polimer pa med sintezo ostane vezan z encimom. Hkrati s sintezo poteka translokacija hitinskega polimera v zunajcelični prostor skozi poro, ki naj bi jo gradili transmembranski heliksi encima (Merzendorfer, 2006, 2011). O začetku nastajanja hitinske verige je še precej neznanega. Nekatere študije, vključno s študijo Horst in sod. (1993), poročajo o tem, da naj bi bil v polimerizacijo hitinske verige vključen začetnik, kovalentno vezan na encim. Predlagajo, da začetnik nastane iz proteina v granuliranem endoplazemskem retikulumu (GER), ki se glikozilira preko membranskega lipida dolihola in se prenese v Golgijev aparat. Z aktivnostjo hitin sintaze naj bi bil pri sintezi hitinske verige dolihol sprejemnik GlcNAc monomerov (Horst in sod., 1993). Vendar pa po naših podatkih novejših raziskav in potrditev o tem ni.

Sintetska pot vseh proteinov, vključno s strukturnimi kutikularnimi proteini, je standardna, od granuliranega endoplazemskega retikuluma (GER), preko Golgijevega aparata in sekrecijskih veziklov do apikalne plazmaleme, kjer v predelih med izboklinami poteka zlivanje veziklov s plazmalemo in eksocitoza v zunajcelični prostor (Moussian, 2013). Po tej poti se sintetizira tudi encim hitin sintaza, ki so ga lokalizirali v membranah Golgijevega kompleksa in znotrajceličnih veziklov in na plazmalemi (pregledno v Merzendorfer in Zimoch, 2003). Predvidoma se hitin sintaza pri žuželkah aktivira z zunajceličnimi proteazami po zlitju vezikla s plazmalemo in se nakopiči na konicah membranskih izboklin (Merzendorfer, 2011). Študije pri žuželkah torej kažejo na to, da organiziranje hitina ter njegovo povezovanje s proteini poteka v zunajceličnem prostoru, za kar je potrebna tudi aktivnost določenih zunajceličnih encimov.

Ultrastrukturne značilnosti citoplazme epitelnih celic, ki jih avtorji izpostavljajo kot tipične za sintezo kutikule, so dobro razvit GER, veliko različnih veziklov v apikalni citoplazmi, prisotnost glikogenskih granul, izrazit Golgijev kompleks in povečana prisotnost mitohondrijev in mikrotubulov v apikalnem delu citoplazme (Goudeau, 1976; Koulish in Klepal, 1981; Ziegler, 1997, Moussian, 2006; Vittori in sod., 2012). Poleg sekrecijskih veziklov, ki izhajajo iz Golgijevega aparata, so opazili tudi plaščne vezikle. Avtorji jih povezujejo z endocitotsko aktivnostjo celice v času levitve, na kar kaže tudi pogosta prisotnost multivezikularnih teles v apikalnem delu citoplazme. Endocitotsko

aktivnost povezujejo z dinamično kontrolo sestave kutikule med odlaganjem (Locke, 2001, 2003), z resorpcijo stare kutikule, s kontrolo sestave levitvene tekočine in z vračanjem membrane ter plakov iz plazmaleme (Locke in Huie, 1979; Koulish in Klepal, 1981; Ziegler, 1997).

Značilne modifikacije apikalne plazmaleme so poleg plakov tudi citoplazemski izrastki, ki med sintezo kutikule zapolnjujejo porne kanale v kutikuli. Pripisujejo jim različne funkcije, predvsem modifikacijo in dostavo lipidov v epikutikularne sloje ter transport kalcijevih ionov v kutikulo med kalcifikacijo (Locke, 1961; Roer in Dillaman, 1984; Powell in Halcrow, 1985; Compere in Goffinet, 1987b; Compere, 1991; Compere, 1995; Ziegler, 1997; Dillaman in sod., 2005; Moussian, 2013).



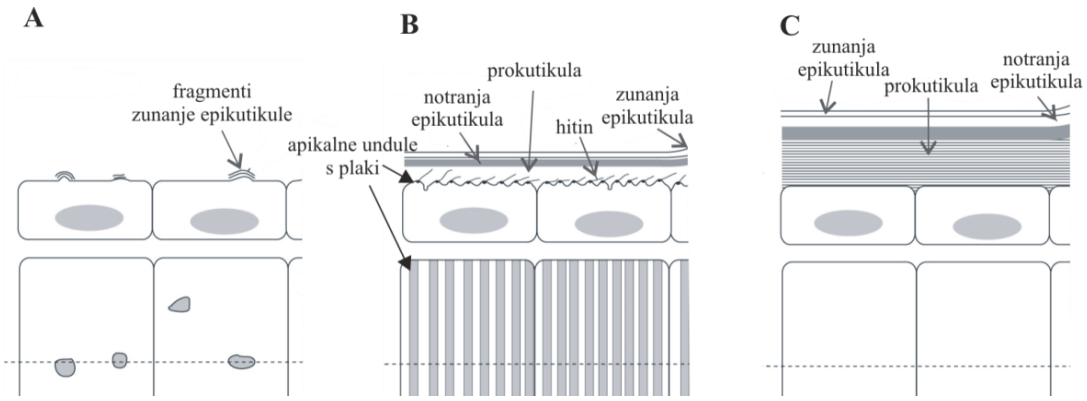
Slika 6: Shematski prikaz biosintetskih poti kutikularnih komponent (prirejeno po Moussian, 2013:175). Prikazane so tri poti mehanizmov tvorbe kutikule: sinteza kutikularnih lipidov, sinteza kutikularnih proteinov in sinteza hitina. Vse poti se začnejo v citosolu in organelih epitelnih celic, nato se komponente prenesejo skozi apikalno plazmalemo v zunajcelični prostor, tam pa se dokončno organizirajo v kutikularni matriks. Lipidi izhajajo iz lipidnih kapelj ali pa iz maščobnih kislin, ki se sintetizirajo v mitohondrijih in gladkem endoplazemskem retikulumu (AER). Nalaganje, organizacija in transport lipidov do njihove končne destinacije naj bi potekala po pornih kanalih kutikule. Sekrecijska pot vseh proteinov je standardna, od granuliranega endoplazemskega retikuluma (GER), preko Golgijevega aparata (ki na shemi ni prikazan) in sekrecijskih veziklov do plazmaleme. Encim hitin sintaza je nakopičen v plakih na konicah membranskih izboklin. Organizacija hitina poteka v zunajceličnem prostoru.

Figure 6: Schematic representation of cuticle production pathways (adopted from Moussian, 2013: 175). Mechanisms of cuticle production can be subsumed in three pathways: cuticle lipid biology, cuticle protein biology and chitin biology. Synthesis of the components starts in the cytosol and organelles of epithelial cell. They transfer across the apical plasma membrane into the extracellular space, where they

finally organize in the cuticular matrix. Lipids are provided by lipid droplets or by fatty acids, synthesized in mitochondria and the smooth endoplasmatic reticulum (AER). Lipid deposition, organization and transport to their final destination probably involve transfer through the pore canals in the cuticle. All proteins synthesize via canonical secretory pathway, from granular endoplasmatic reticulum (GER), across Golgi apparatus (not shown in the sheme) and secretory vesicles to the plasma membrane. Chitin synthase is localized in plaques at the tips of membrane protrusions. Chitin organization occurs in the extracellular space.

1.1.4.2 Diferenciacija kutikule členonožcev med embrionalnim razvojem

Diferenciacija hitinskih matriksov med embrionalnim razvojem je poznana predvsem pri žuželkah (Dorn, 1976; Ziese in Dorn, 2003; Konopova in Zrzavy, 2005; Moussian in sod., 2006), medtem ko je o zgodnjih fazah tvorbe hitinskih matriksov pri rakih zelo malo podatkov. Pri vinski mušici (*D. melanogaster*) se eksoskeletna kutikula tvori v drugi polovici embriogeneze in je ob koncu embrionalnega razvoja diferencirana in v osnovi morfološko zelo podobna kutikuli odraslih (sl. 7). Pred nastankom prave kutikule epidermis apikalno izloči zunajcelični matriks, ki ga pri drugih žuželkah imenujejo tudi embrionalna kutikula. Ta matriks je sestavljen iz neurejenega materiala, ki ga pokriva tanek elektronsko gosti sloj. Moussian in sod. (2006) diferenciacijo kutikule pri vinski mušici razdelijo na tri faze. V prvi fazi se organizirajo trije glavni sloji. Najprej se na konicah neenakomerno razporejenih izboklin apikalne plazmaleme začne nalaganje zunanje epikutikule (pri žuželkah imenovana 'envelope'). Nastanejo fragmenti zunanje epikutikule, ki se nato povežejo v enoten sloj. Apikalna plazmalema epidermalnih celic se preoblikuje v pravilno razporejene izbokline, ki potekajo vzdolžno na celično površino . Imenujejo jih apikalne undule. Takoj po nastanku enotnega sloja zunanje epikutikule se začne nalaganje prokutikule, ki kasneje poteka hkrati z nalaganjem notranje epikutikule. V drugi fazi diferenciacije kutikule se kutikularni sloji postopoma debelijo in diferencirajo, v zadnji fazi pa v kutikuli poteče še organizacija hitinskih lamel in sklerotizacija, s čimer kutikula pridobi mehanske lastnosti (Moussian in sod., 2006).



Slika 7: Shematski prikaz poteka diferenciacije eksoskeletne kutikule in preoblikovanje apikalne plazmaleme epidermalnih celic pri embriju vinske mušice *Drosophila melanogaster* (prirejeno po Moussian in sod., 2010). Zgornje sheme prikazujejo prereze epidermalnih celic med diferenciacijo kutikule, spodnje sheme pa pogled na površino teh epidermalnih celic. **A**: Nalaganje zunanje epikutikule ('envelope') se začne po fragmentih na konicah izboklin apikalne plazmaleme. **B**: Organizirajo se trije kutikularni sloji: zunanja epikutikula, notranja epikutikula in prokutikula. Apikalna plazmalema je oblikovana v vzdolžne izbokline, apikalne undule. Na grebenih izboklin se nahajajo plaki, kjer poteka sinteza hitinskih vlaken, med njimi pa so predeli zlivanja veziklov s plazmalemo. **C**: Notranja epikutikula in prokutikula se hkrati debelita, prokutikula se diferencira v podsloje. Po končani diferenciaciji kutikule se apikalna plazmalema zravna.

Figure 7: Schematic representation of cuticle differentiation and the dynamics of the apical plasma membrane of the epidermal cells in the *Drosophila melanogaster* embryo (adopted from Moussian et al., 2010). The upper scheme illustrates sections of the epidermal cells during cuticle differentiation. The lower sheme provides a top view of the epidermal cells. **A:** The outer epicuticle ('envelope') is deposited as the fragments at the tips of the apical plasma membrane bulges. **B:** The three cuticular layers are organized: the outer epicuticle, the inner epicuticle and the procuticle. Apical plasma membrane is modified in regular longitudinal corrugations, the apical undulae. The crests of the corrugations carry chitin synthesizing plaques, and between them are the sites where vesicles fuse with plasma membrane. **C**: The inner epicuticle and procuticle are thickened and differentiated into the sublayers. When cuticle differentiation ceases, the apical plasma membrane flattens.

Pri rakih največ podatkov o eksoskeletni kutikuli med razvojem izhaja iz študij deseteronožcev (Decapoda), škrgonožcev (Branchiopoda) in postranic (Amphipoda). Zgodnje študije levitvenega cikla ličink so omejene na histološke raziskave in opazovanja *in vivo* intaktnih, nefiksiranih larv (Freeman in Costlow, 1980; Anger, 1983; Snyder in Chang, 1986). Za zgradbo embrionalnih kutikul in potek levitvenih ciklov so na voljo študije *in vivo* opazovanja embrijev (Belk, 1987; Helluy in Beltz, 1991), le redko pa zajemajo ultrastrukturne analize. Ultrastruktura embrionalnih površinskih matriksov je opisana v nekaterih starejših študijah: pri škrgonožcu *Artemia salina* (Morris in Afzelius, 1967), pri enakonožcu *Hemioniscus balani* (Goudeau, 1976) in pri desetoronožcih *Carcinus maenas* (Goudeau in Lachaise, 1983) in *Palaemonetes pugio* (Glas in sod., 1997). Novejša ultrastrukturna raziskava je narejena pri vodni postranici

Parhyale hawaiensis (Havemann in sod., 2008). Sprva je epidermis pokrit s tankim slojem zunajceličnega matriksa, kasneje pa nastane debelejši matriks, ki ga imenujejo embrionalna kutikula in je morfološko podoben zgodnjemu matriksu pri vinski mušici. Pod tem matriksom se v zadnjih stadijih embrionalnega razvoja izoblikuje kutikula, ki ima ob koncu embriogeneze že značilnosti kutikule odraslih živali (Havemann in sod., 2008).

Povzeto po teh študijah za členonožce v splošnem velja, da embrionalni epidermis na površino izloči po dva do pet zunajceličnih matriksov. Med njimi je zadnji matriks, ki se oblikuje pred izleganjem embrija, kutikula ličinke, vsi prejšnji embrionalni matriksi pa se pred izleganjem odluščijo s površine embrija.

1.1.5 Povezave eksoskeleta z mišicami

Mišičnoskeletni sistem, ki obsega mišice, skelet in povezovalna tkiva (kite oziroma specializirane epitelne celice), je učinkovit sistem za gibanje in stabilnost celotnega organizma. Pri členonožcih so mišice povezane z eksoskeletno kutikulo. Na mestu teh povezav so epidermalne celice specializirane v posebne povezovalne celice ('tendon cells' / tenocite), ki imajo podobne funkcionalne lastnosti kot kite pri vretenčarjih (Bitsch in Bitsch, 2002; Schweitzer in sod., 2010). Apikalno se te celice mehansko povezujejo s kutikularnim matriksom, njihove bazalne površine pa se pritrjajo na spodnje mišične celice. Tenociti vsebujejo obsežne snope mikrotubulov, ki potekajo v apikalno-bazalni smeri in omogočajo mehanske lastnosti, potrebne za prenašanje mišične kontrakcije. Na apikalni celični membrani so oblikovani stiki, preko katerih je eksoskelet z navpičnimi znotrajkutikularnimi vlakni pritrjen na epidermis. Na bazalni strani mikrotubuli segajo do znotrajceličnega elektronsko gostega materiala, ki se nahaja pod bazalno plazmalemo (bazalni adherentni stik). Stik med mišično celico in tenocitom poteka vzdolž celotne površine bazalne membrane tenocita, ki je nagubana v vzorec cikcak in se natančno ujema z gubami apikalne plazmaleme mišične celice. Z gubami povečana površina povezave med celicama poveča mehansko trdnost stika. Na drugi strani stika, v mišični celici, so na elektronsko gost material pod plazmalemo pritrjeni aktinski in miozinski filamenti mišične celice. Obe celični površini s spodaj ležečim elektronsko gostim materialom in tankim slojem zunajceličnega matriksa v ozkem medceličnem prostoru sestavljajo kompleksno mehansko povezavo med mišično celico in tenocitom. Takšna ultrastrukturna organizacija stika je pomembna za ustrezen prenos sile mišične kontrakcije, zunajcelični matriks v medceličnem prostoru pa zagotavlja elastične lastnosti stika. Mišice so na ta način s kompleksno mrežo citoskeletnih elementov in medceličnih stikov mehansko povezane z epidermisom in eksoskeletom.

Med nastajanjem nove kutikule med razvojem in kasneje med obnavljanjem eksoskeleta, ko sočasno potekata razgrajevanje starega kutikularnega matriksa in tvorba nove kutikule, poteka intenzivna reorganizacija teh povezav. Vzpostaviti se morajo povezave novega eksoskeleta z mišicami, kar je pomembno za vzdrževanje integritete integumenta in zmožnosti gibanja med razvojem in med potekom menjave eksoskeleta. Poleg tega imajo mišične celice pomembno vlogo med obnavljanjem kutikule, saj omogočajo gibanje živali, da se lahko izvleče iz starega eksoskeleta. Ultrastrukturna organizacija povezav eksoskeleta z mišicami pri odraslih rakih enakonožcih v levitvi in pri marzupijskih razvojnih stadijih še ni raziskana. Diferenciacija teh specializiranih povezovalnih kompleksov med eksoskeletom in mišicami je ultrastrukturno in molekulsko precej poznana pri embriju modelne vinske mušice *Drosophila melanogaster* (Volk, 1999; Schweitzer in sod., 2010). Pri rakih so študije teh povezav redke, ultrastrukturno so raziskane med levitvijo odraslih živali pri nekaterih vodnih rakih (Buchholz in Buchholz, 1989; Yamada in Keyser, 2009).

1.2 RAZISKOVALNA VPRAŠANJA IN HIPOTEZE

1.2.1 Cilji in raziskovalna vprašanja

Osrednji del doktorskega dela je namenjen preučitvi strukturne diferenciacije eksoskeletne kutikule med razvojem embrijev in ličink v valilniku raka enakonožca *Porcellio scaber*. Navadni prašiček ali mokrica vrste *P. scaber* je primeren modelni organizem iz skupine kopenskih rakov enakonožcev (Oniscidea) za študije embrionalnega razvoja. Znanje o morfogenezi kutikule pri členonožcih v največji meri izhaja iz raziskav nemineraliziranega hitinskega matriksa pri žuželkah, predvsem pri modelnem organizmu *Drosophila melanogaster*. Študije kutikule med razvojem rakov so večinoma omejene na histološke raziskave ali *in vivo* opazovanja intaktnih embrijev in ličink, ultrastrukturnih podatkov pa je zelo malo. Pomemben del naloge je pridobitev podatkov o zgodnji kalcifikaciji kutikule pri razvojnih stadijih mokrice *P. scaber*. Proučevanje zgodnjih faz kalcifikacije bioloških tkiv je sicer področje intenzivnih raziskav, vendar pa večinoma ne poteka na razvojnih stadijih. Kalcifikacija kutikule marzupijskih mank še ni poznana. V širšem kontekstu so rezultati tega doktorskega dela doprinos novega znanja na področju diferenciacije mineraliziranih hitinskih matriksov.

Kopenski raki enakonožci so uspešno poselili kopno in v zvezi s tem razvili specifične strukturne in fiziološke prilagoditve. Med te spada tudi embrionalni razvoj v specifičnem vodnem okolju valilnika (marzupija) samice in struktura eksoskeletne kutikule. V okviru raziskav prilagoditev na kopenski način življenja in razvoja v valilniku je del naloge namenjen tudi raziskavam ultrastrukture jajčnih ovojnic rakov enakonožcev rodu *Porcellio*, specializiranih matriksov, ki med razvojem obdajajo in ščitijo embrij.

Specifični cilji te naloge so:

- opisati in pojasniti funkcionalno ultrastrukturo apikalnih zunajceličnih matriksov epidermisa pri embrijih in marzupijskih mankah;

- analizirati sestavo apikalnih matriksov epidermisa, s poudarkom na lokalizaciji in analizi distribucije hitina kot osnovne organske komponente kutikularnega matriksa, lokalizaciji kalcificiranega matriksa ter analizi elementne in mineralne sestave kutikule;

- identificirati ultrastrukturne značilnosti epidermalnih celic med diferenciacijo v različnih razvojnih stadijih, v povezavi s sintezo zunajceličnega matriksa, s poudarkom na modifikacijah apikalne plazmaleme, organiziranostjo apikalne citoplazme in prisotnosti medceličnih stikov;

- pojasniti ultrastrukturno organizacijo mehanskih povezav med kutikulo in mišicami pri embrijih in marzupijskih mankah.

Pridobljene rezultate smo primerjali in vrednotili predvsem glede na rezultate raziskav diferenciacije nemineraliziranega hitinskega matriksa in epidermisa pri žuželkah ter glede na dosedanje poznavanje nastajanja novega mineraliziranega hitinskega matriksa pri odraslih rakih med levitvijo.

Ena ključnih prilagoditev terestričnih vrst oniscidov je tudi prebavna cev, ki je v celoti pokrita s kutikulo. Primerjalno z diferenciacijo eksoskeletne kutikule smo analizirali tudi diferenciacijo kutikule črevesa med razvojem embrijev in ličink v valilniku.

1.2.2 Raziskovalne hipoteze

Predvidevamo, da sta jajčni ovojnici (horion in vitelinska membrana) tanjši in manj kompleksno zgrajeni kot pri žuželkah, ker poteka razvoj enakonožcev v valilniku, razvoj žuželk pa v zunanjem okolju.

Predvidevamo, da so zgodnje faze sinteze apikalnega matriksa epidermisa pri raku enakonožcu *P. scaber* podobne kot pri drugih členonožcih, ker gre pri vseh za tvorbo hitinskega matriksa. Glede na podatke iz študij drugih rakov in iz študij žuželk se pred nastankom značilne eksoskeletne kutikule členonožcev tvori strukturno preprostejši zunajcelični matriks, sestavljen iz tanke elektronsko goste lamine in neurejenega materiala pod njo.

Za kasnejše faze oblikovanja zunajceličnega matriksa epidermisa pričakujemo, da bomo ugotovili podobnosti s sintezo kutikularnega matriksa odraslih živali v levitvi, ker gre za sintezo enake strukture - eksoskeletne kutikule. Poleg podobnosti pa predvidevamo tudi razlike, ker:

- embrionalni matriksi nastajajo v drugačnem mikrookolju, to je pod jajčno ovojnico, medtem ko tvorba kutikule odraslih poteka v levitvenem prostoru pod staro kutikulo;
- so funkcije matriksov različne: pri embrijih imajo predvidoma pomembno transportno vlogo in delno tudi zaščitno, medtem ko kutikula odraslih rakov služi predvsem zaščiti, opori in gibanju živali. Pri marzupijskih ličinkah kutikula verjetno postopoma prevzema zaščitno in oporno funkcijo eksoskeleta, predvsem zaščito pred osmotskimi in ionskimi spremembami v marzupijskem okolju zaradi odsotnosti jajčnih ovojnic ter sposobnost gibanja mank, ki omogoča njihovo sprostitev v zunanje okolje.

Glede na podatke iz študij marzupijskih mank rakov enakonožcev pričakujemo, da se kalcifikacija hitinskega matriksa začne pred sprostitvijo mank v zunanje okolje. V literaturi je poročilo, da se koncentracija kalcija v srednjih fazah razvoja marzupijskih mank znatno poveča, vendar pa kalcija niso natančneje lokalizirali.

Glede na to, da med razvojem prihaja do precejšnih morfoloških sprememb, povezanih z rastjo (velikost med razvojem od oplojenega jajčeca do pozne marzupijske manke se poveča približno trikrat), predvidevamo, da se epidermalni matriksi v tem času tudi obnavljajo.

V okviru raziskav ultrastrukturnih značilnosti epidermalnih celic med diferenciacijo v različnih razvojnih stadijih in sintezo zunajceličnega matriksa pričakujemo pojav mikrovilom podobnih izboklin z elektronsko gostimi konicami na apikalni plazmalemi, intenzivneje razvite strukture, ki so udeležene pri sintezi matriksa v apikalni citoplazmi (predvsem granulirani ER, Golgijev aparat in sekrecijski vezikli) in vzpostavitev medceličnih stikov v subapikalnem področju plazmaleme. Ker ni podatkov, na osnovi katerih bi lahko predvideli, v katerih razvojnih stadijih se pojavijo navedene ultrastrukturne značilnosti, smo najprej analizirali manke in potem zgodnejše embrionalne stadije.

Predvidevamo, da se povezave med eksoskeletom in mišicami vzpostavijo že med razvojem v valilniku, ker smo opazili intenzivnejše gibanje marzupijskih ličink in ker je znano, da se ličinke levijo kmalu po sprostitvi iz valilnika.

Predvidevamo, da se v zgodnjih razvojnih obdobjih apikalni matriks črevesnih epitelnih celic ne bo bistveno razlikoval od epidermalnega matriksa, saj gre v obeh primerih za sintezo hitinskega matriksa *de novo*. V poznejših razvojnih obdobjih pa bodo opazne izrazitejše strukturne razlike med obema matriksoma zaradi različnih funkcij. Kutikula črevesa ima namreč pri funkcionalnem črevesu vlogo pri predelavi, transportu in absorpciji hrane.

2 ZNANSTVENA DELA

2.1 OBJAVLJENA ZNANSTVENA DELA

2.1.1 Jajčne ovojnice in obnavljanje kutikule pri embrijih in marzupijskih mankah mokrice rodu *Porcellio*

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

Polona Mrak, Nada Žnidaršič, Magda Tušek-Žnidarič, Waltraud Klepal, Daniela Gruber in Jasna Štrus
Zookeys, 2012, 176: 55-72

V tem članku smo predstavili strukturo jajčnih ovojnic pri embrijih raka enakonožcu rodu *Porcellio*. Poročamo tudi o obnavljanju eksoskeletne kutikule med razvojem znotraj valilnika. Razvoj v valilniku ali marzupiju, s tekočino napolnjeni vrečasti strukturi na trebušni strani samice, je pomembna prilagoditev kopenskih rakov enakonožcev na kopenske habitate. Embrije in ličinke manke v valilniku pokrivajo različni površinski matriksi, ki imajo zaščitno vlogo in se med razvojem obnavljajo. V raziskavi smo s presevnim in vrstičnim elektronskim mikroskopom proučili ultrastrukturo jajčnih ovojnic pri zgodnjem, srednjem in poznem embriju ter ultrastrukturne značilnosti eksoskeletne kutikule, s poudarkom na analizi morfoloških znakov obnavljanja pri poznem embriju pred izleganjem in pri marzupijskih ličinkah mankah. Embrij pokrivata dve jajčni ovojnici, zunanji horion, ki je prisoten pri zgodnjih in srednjih embrijih, ter notranja vitelinska membrana, ki je prisotna do konca embrionalnega razvoja. Jajčne ovojnice mokrice so v primerjavi z jajčnimi ovojnicami vinske mušice (*Drosophila*) tanjše in imajo relativno preprosto ultrastrukturno arhitekturo. Te razlike so verjetno posledica različnih okoljskih razmer embrionalnega razvoja, saj ta pri vinski mušici poteka neodvisno od samice v zunanjem okolju. V pozni embriogenezi epidermalne celice tvorijo kutikulo. Kutikula se v nadaljnjem razvoju obnavlja v povezavi z rastjo razvijajočih se embrijev in ličink. Pri poznem embriju tik pred izleganjem in pri marzupijski manki je ultrastruktura kutikule v glavnem podobna kutikuli odraslih živali. Epikutikula je tanka in homogena. Značilna ureditev hitinsko-proteinskih vlaken in gosti zunanji sloj v eksokutikuli sta pri poznem embriju pred izleganjem težje razločna, pri marzupijski manki pa bolj izrazita. Endokutikulo v obeh stadijih gradijo izmenični elektronsko gosti in elektronsko svetli podsloji, skoznjo pa potekajo porni kanali. Že v stadiju poznega embrija pred izleganjem so diferencirane tudi kutikularne luske in senzile. Razlike med kutikulo odraslih živali in kutikulo embrija ter marzupijske manke se kažejo v debelini, ultrastrukturi in mineralizaciji kutikule. Pri poznem embriju pred izleganjem in pri marzupijski manki smo opazili morfološke znake obnavljanja kutikule, in sicer odmik kutikule od epidermisa in delno razgradnjo endokutikule ter pri manki tudi sintezo nove kutikule.

ZooKeys 176: 55–72 (2012)
doi: 10.3897/zookeys.176.2418
www.zookeys.org

RESEARCH ARTICLE

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

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Academic editor: *S. Sfenthouraki* | Received 22 November 2011 | Accepted 26 January 2012 | Published 20 March 2012

Citation: Mrak P, Žnidaršič N, Tušek-Žnidarič M, Klepal W, Gruber D, Štrus J (2012) Egg envelopes and cuticle renewal in *Porcellio* embryos and marsupial mancas. In: Štrus J, Taiti S, Sfenthourakis S (Eds) Advances in Terrestrial Isopod Biology. ZooKeys 176: 55–72. doi: 10.3897/zookeys.176.2418

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 mancas. 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 ultrastuctural 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 mancas. Cuticle structure and renewal in prehatching late-stage embryos and marsupial mancas exhibit main features of cuticle in adults. Epicuticle is thin and homogenous. The characteristic arrangement of chitin-protein fibers and the dense distal layer in exocuticle are hardly discernible in prehatching embryo and distinct in marsupial mancas. 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 mancas 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

Introduction

The unique feature of embryonic development in isopod and amphipod crustaceans (Peracarida) is its location in the brood pouch on the ventral side of female body (marsupium). The marsupium has likely been of a great adaptive significance in the colonization of land by crustacean species as it allows embryonic development to occur in aqueous environment within the protected chamber (Hornung 2011, Warburg 2011). Two main types of marsupium are distinguished in Oniscidea, the amphibian or open type and the terrestrial or closed type marsupium (Hoese and Janssen 1989). In the former, marsupium is partly open and water from the external environment can pass into the marsupium. In the closed type, marsupium is a watertight structure, provisioned only with fluid from the mother. Marsupial fluid is persistently osmotic and ionic regulated, probably via transporting epithelia of segmental cotyledons, which hang down from the overlying sternites (Surbida and Wright 2001). Depending on the level of maternal control of the marsupial environment, osmotic tolerance of the embryos and marsupial mancas is of adaptive importance. Protective envelopes between embryo/manca and marsupial fluid function against potential physiological stresses, including osmotic and ionic variation and desiccation in marsupial environment (Charmantier and Charmantier-Daures 2001, Surbida and Wright 2001). Ontogenetic development of terrestrial isopod crustacean *Porcellio scaber*, from released fertilized eggs to embryos and marsupial mancas, occurs in the terrestrial type marsupium. Intramarsupial development of *P. scaber* lasts approximately 35 days (Milatovič et al. 2010), and during development individuals in different stages are coated by different protective envelopes - egg envelopes (chorion and vitelline membrane) and cuticle. During growth of embryos and mancas egg envelopes are shed and cuticle is renewed.

Two egg envelopes, the outer chorion and the inner vitelline membrane, are produced during oogenesis by the somatic follicle cells of the female reproductive system and cover the embryo until the transition to the late-stage embryo and hatching, respectively. Arthropods evolved egg envelopes of different morphologies and complexities as a consequence of embryonic development in different environments. The data on egg envelopes (chorion and vitelline membrane) structure derive mainly from the studies on the fruit fly *Drosophila melanogaster*, as this model species offers wide opportunities for genetic studies (Margaritis et al. 1980, Jagadeeshan and Singh 2007), while there are no data on isopod chorion and vitelline membrane ultrastructure. In contrast to *Porcellio scaber* embryonic development in the female protected environment, *Drosophila* embryonic development takes place independently of the female. The egg envelopes of *Drosophila*, especially the chorion, are structurally complex and their proteins show signs of evolving under selection by ecological factors. At the morphological level, differences in egg envelopes surfaces between specialists and generalists of *Drosophila* species were found, particularly in the surface ridges and surface porosity (Jagadeeshan and Singh 2007).

Cuticle is the innermost protective layer, formed later during intramarsupial development and is produced by hypodermal cells of the embryo. In adult arthropods the

cuticle is a complex hierarchically structured extracellular matrix, consisting of chitin, proteins and lipids, hardened mostly by mineralization in crustaceans in contrast to insect cuticle which is only sclerotized. It comprises the distal epicuticle, the exocuticle in the middle and the proximal endocuticle. Several reports were published on cuticle structure in adult isopods, mostly in *Porcellio scaber* (Price and Holdich 1980, Štrus and Compere 1996, Ziegler 1997, Glötzner and Ziegler 2000, Štrus and Blejec 2001, Hild et al. 2008, Hild et al. 2009). The thin and non-calcified epicuticle is composed mainly of lipoproteins and consists of thinner 5-layered outer epicuticle and thicker inner epicuticle. The exocuticle, comprising sublayers of chitin–protein fibers arranged in characteristic pattern and the endocuticle, consisting of lamellar chitin–protein sublayers, are calcified. In addition, the thin non-calcified membranous layer lies between the endocuticle and the epithelial cells. The ultrastructure and composition of the cuticle in isopod embryos and marsupial mancas have not been studied in detail, while cuticle structure is well described in *Drosophila melanogaster* embryos (Locke 2001, Payre 2004, Moussian et al. 2006, Moussian 2010). Fully formed cuticle in *Drosophila* embryo is organized in distinct horizontal layers. The distal lipoprotein epicuticle is subdivided in the outer thin epicuticle (cuticulin layer) and the inner thick epicuticle and the proximal chitin-protein procuticle consists of several lamellae.

Cuticle renewal is related to growth in arthropods. In crustaceans molting frequently recurs during adult life. Isopod crustaceans molt in two phases, separately molting posterior and anterior parts of the body. Molt cycle begins with premolt stage, when remarkable morphological changes of the integument occur. The old cuticle separates from the underlying epithelium (apolysis). Epithelial cells secrete a new cuticle, starting with the epicuticle and followed by pre-ecdysal exocuticle. The old and the new cuticles are separated by an extracellular compartment, the ecdysal space, containing different material involved in cuticle renewal. At molting the old cuticle is shed and the new cuticle is further produced, forming post-ecdysal endocuticle. Postmolt stage is marked by soft body surface with progressive hardening of the exoskeleton until the intermolt stage.

In this study we present new data on the ultrastructural architecture of egg envelopes, including chorion and vitelline membrane, and on the ultrastructural characteristics of cuticle renewal in embryos and marsupial mancas of isopod crustaceans *Porcellio scaber* and *Porcellio dilatatus*. Comparison of envelopes structure in terrestrial crustaceans and insects will bring new insights into the protective role of egg envelopes and cuticle renewal in developing embryos of these two terrestrial arthropod groups with different developmental strategies.

Methods

Animals were maintained and bred in a laboratory culture. Staging system of *P. scaber* ontogenetic development, based on morphological characteristics of embryos and marsupial mancas, was used in this study (Milatovič et al. 2010). Embryos of *P. dilatatus* were used in scanning electron microscopic studies of egg envelopes.

The embryos and marsupial mancas of different developmental stages are shown in Figures 1A, 2A, 3A, 4A and 5A-C in the Results. The term early-stage embryo is used for embryos with large amount of yolk mass in the central part and no visible limb buds. A mid-stage embryo has visible developing limb buds and two midgut glands primordia, which enclose yolk. After bending ventrally and shedding of chorion, embryos are termed late-stage embryos. Prior to hatching swelled embryo inside the vitelline membrane is described as a prehatching late-stage embryo. When late embryos hatch from the vitelline membrane they become marsupial mancas. The progress of development of *P. scaber* marsupial mancas is characterized by the following morphologically discernible modifications: reduction of the midgut glands size due to yolk consumption, increase in exoskeleton pigmentation, enlargement of body size and pronounced locomotion (Wolff 2009, Milatovič et al. 2010). In previous studies stages of marsupial mancas were not precisely determined. For this reason and according to the morphological characteristics listed above, we determined three sequential developmental stages of marsupial mancas, early-stage, mid-stage and late-stage marsupial mancas. The term early-stage manca is used for 1.5 – 1.6 mm long mancas with no or very little locomotion inside the marsupium, with scarce chromatophores on the body surface and with the midgut yolk extending into the pleon. Mid-stage mancas are 1.7 - 1.8 mm long, with darker pigmentation on the head region and tergites and with the midgut yolk only partly extending into the pleon. Late-stage mancas are 1.9 - 2.0 mm long, with pronounced locomotion of the whole body and pereopods. The yolk in the midgut extends only to the end of the pereon.

Embryos and mancas at different stages of development were isolated from the marsupium and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). Prior to fixation, the egg envelopes of embryos were either carefully perforated with a thin needle or removed. After washing in cacodylate buffer, the samples were postfixed in 1% osmium tetroxide for 2 hours, washed again and dehydrated in a graded series of ethanol.

Embryos and mancas of *P. scaber* for light microscopy (LM) and transmission electron microscopy (TEM) were embedded in Agar 100 resin. Prior to embedding, mancas were perforated with a thin needle for better infiltration of resin. Semithin sections were made with a glass knife, stained with Azure II - Methylene Blue and imaged by Zeiss AxioImager Z.1 light microscope, equipped with a HRC Axiocam camera. Ultrathin sections were made with a Reichert Ultracut S ultramicrotome (Leica), contrasted with 4% uranyl acetate for 10 minutes and 10% lead citrate for 5 minutes and inspected with a Philips CM100 transmission electron microscope, equipped by BioScan 792 camera (Gatan).

After dehydration in a graded series of ethanol and in acetone, *P. dilatatus* specimens for scanning electron microscopy (SEM) were transferred into hexamethyldisilazane (HMDS) to perform chemical drying. Mounted specimens were coated with gold and observed with scanning electron microscopes (Philips XL20 and Philips XL30).

For histochemical detection of calcified tissue, mancas of *P. scaber* were isolated from the marsupium and fixed in 3.7% formaldehyde in 0.1 M cacodylate buffer (pH 7.2). Specimens were washed in cacodylate buffer and embedded in tissue freezing

medium (Jung). Transversal sections (10 μm) were cut with a Leica CM1850 cryostat at –18°C and stained with Alizarin red S solution in 0.2 M Trihydroxylmethyl aminomethane (Tris) - HCl buffer (pH 9). Cuticle of adult *P. scaber* was used as a positive control. Sections were imaged by Zeiss AxioImager Z.1 light microscope, equipped with a HRC Axiocam camera.

Results

Ontogenetic development of *Porcellio scaber* in the marsupium, from released fertilized eggs to marsupial mancas, was recently described morphologically (Wolff 2009, Milatovič et al. 2010), but the issue of protective envelopes was not addressed specifically. We present here the ultrastructural architecture of chorion, vitelline membrane and cuticle in embryos and marsupial mancas of isopod crustaceans *P. scaber* and *P. dilatatus*.

Ultrastructure of chorion and vitelline membrane

Both egg envelopes, distal chorion and proximal vitelline membrane, surround early-stage (Figs 1A, B, C, E) and mid-stage embryos (Figs 2A, B). Chorion has a similar appearance in both stages. It is a one-layered envelope, separated from the embryo surface and is approximately 500 nm thick. Ultrastructurally chorion consists of an electron dense matrix with sparse electron lucent "lacunae" (Figs 1D, 2C, 2D).

The vitelline membrane surrounds embryos throughout the whole developmental period (Figs 1A, 1 B, 1E, 2A, 2B, 3A, 3B, 3E, 4A). It maintains the same thickness of approximately 200 nm from early-stage till late-stage embryo. It is closely apposed to the embryo surface in early-stage embryos (Figs 1B, D, F), while it is slightly detached from the embryo surfaces of mid-stage embryos (Figs 2B, E, F) and late-stage embryos (Figs 3B, C, F, G). Above the embryo limb buds a wider space appears between embryo surface and vitelline membrane due to intense cell rearrangement during limb buds formation (Fig. 2B). The vitelline membrane consists of a thick proximal homogenous electron dense matrix superposed by a thin middle electron dense layer and a superficial corrugated lucent layer (Figs 1F, 2E, 2F, 3C). In non-osmicated specimens of late-stage embryo, the main thick layer is evidently lighter and superficial layer is not discerned (Fig. 3D). Between the outer embryo surface and the vitelline membrane of late-stage embryo a network of fibers was observed by SEM, presumably functioning as connective elements (Figs 3F, G).

Structure and renewal of cuticle

In late-stage embryo the embryo surface is covered with a homogenous extracellular matrix (Figs 3C, F, G). The prehatching late-stage embryo is the earliest developmental

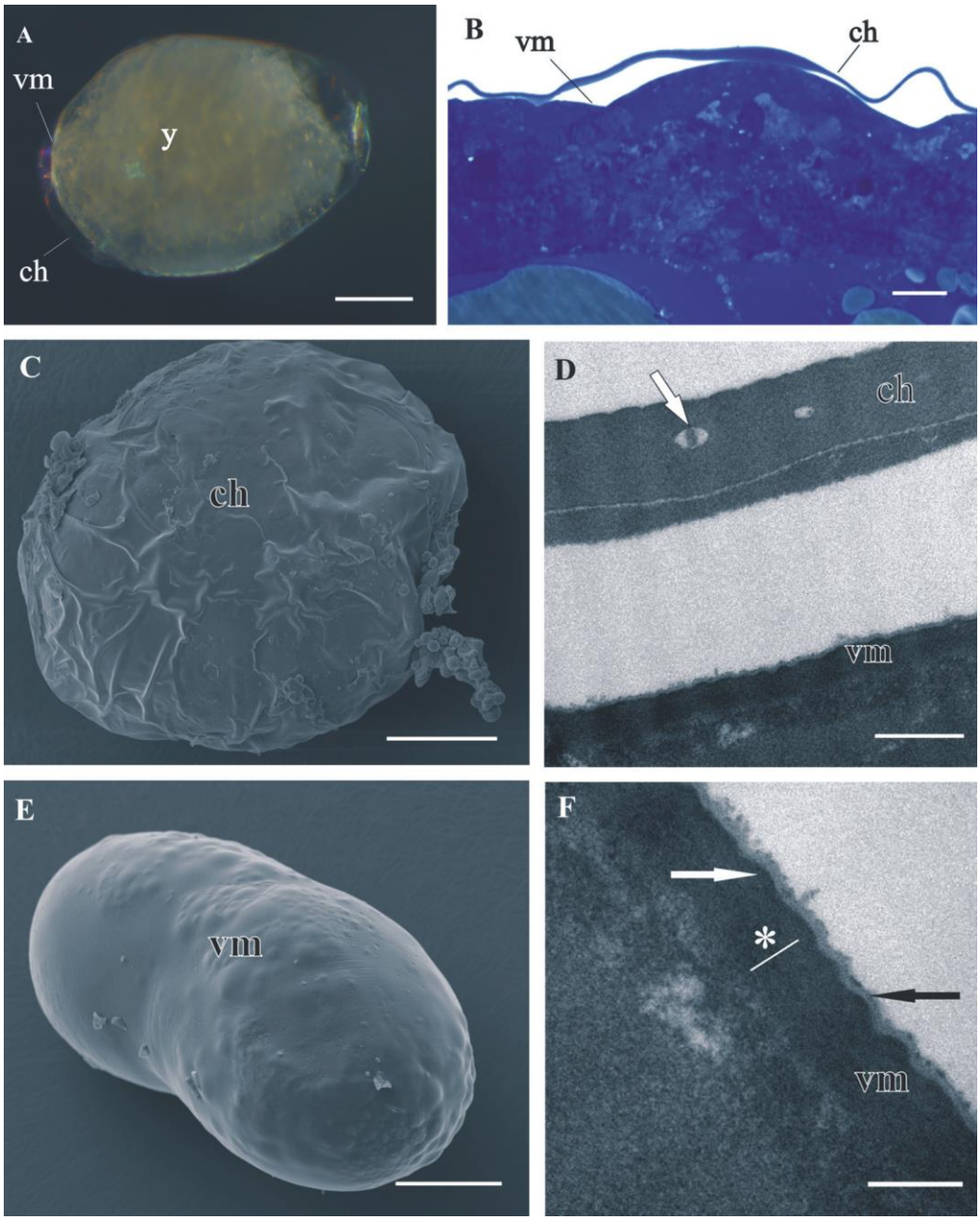

Figure 1. Structure of distal chorion (**ch**) and proximal vitelline membrane (**vm**), covering *P. scaber* **A, B, D, F** and *P. dilatatus* **C, E** early-stage embryo. **A** The early-stage embryo with large amount of yolk (**y**) and no visible limb buds. **B** Semithin section of the embryo peripheral region. Chorion is separated from the embryo surface. The vitelline membrane is closely apposed to the embryo surface. **C** SEM micrograph of the early-stage embryo. The outer egg envelope, chorion, is visible. **D** TEM micrograph of one-layered chorion, including electron lucent "lacunae" (white arrow). There is a layer of artificially spilt yolk underneath the chorion. **E** SEM micrograph of the early-stage embryo. Chorion is artificially removed and the inner egg envelope, vitelline membrane, is exposed. **F** TEM micrograph of vitelline membrane, composed of three layers: main proximal homogenous layer (*), thin middle electron dense layer (white arrow) and superficial corrugated lucent layer (black arrow). Bars: **A, C, E** 200 μm; **B** 10 μm; **D** 0.5 μm; **F** 200 nm.

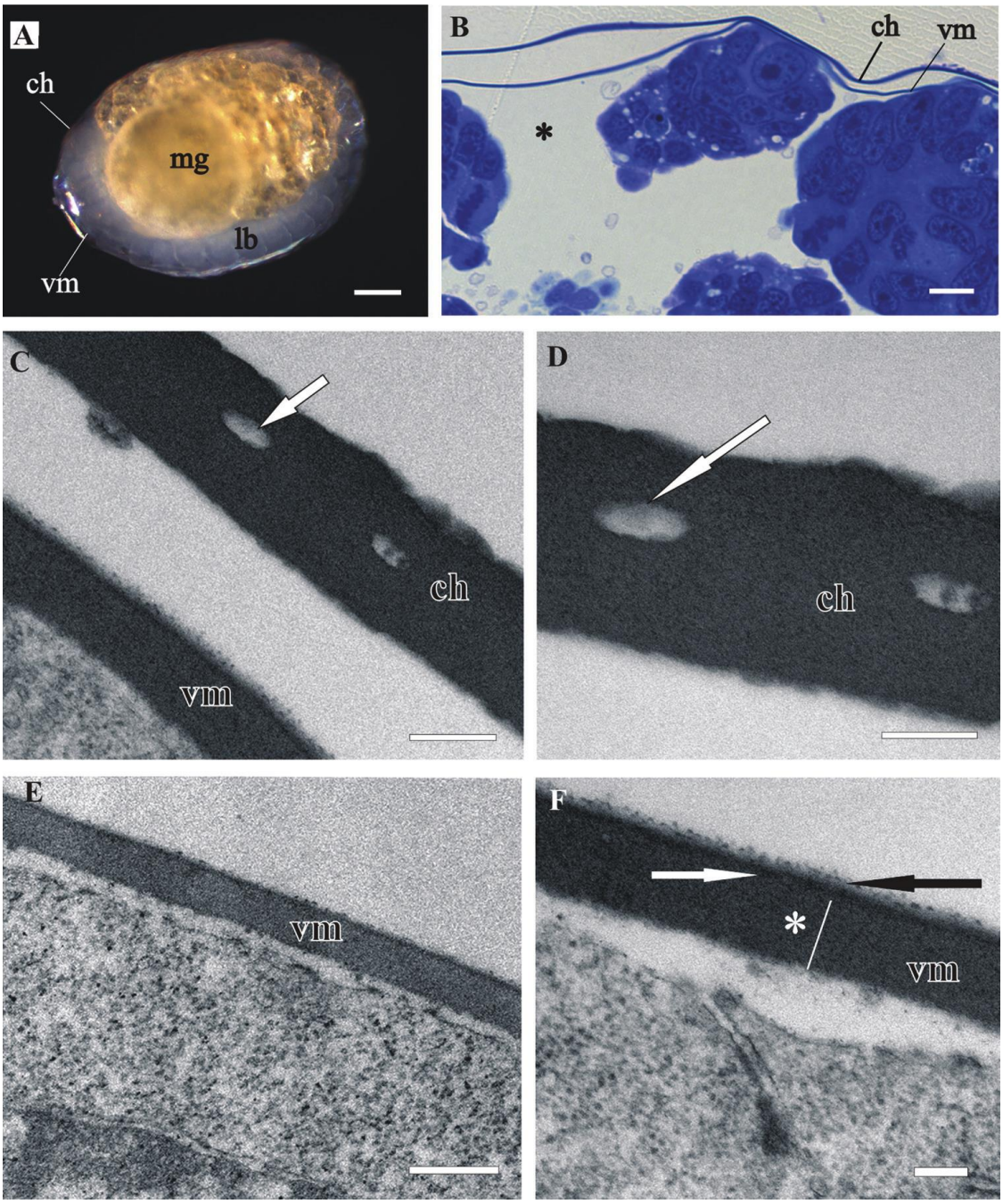

Figure 2. Structure of distal chorion (**ch**) and proximal vitelline membrane (**vm**), covering *P. scaber* mid-stage embryo. **A** The mid-stage embryo with visible limb buds (**lb**) and midgut glands primordia (**mg**). **B** Semithin section of the embryo peripheral region. Chorion is separated from the embryo surface. The vitelline membrane is slightly detached from the embryo cells; * - a wider space between embryo surface and vitelline membrane. **C, D** TEM micrographs of one-layered chorion, including electron lucent "lacunae" (white arrow). **E, F** TEM micrographs of vitelline membrane, composed of three layers: main proximal homogenous layer (*), thin middle electron dense layer (white arrow) and superficial corrugated lucent layer (black arrow). Bars: **A** 200 μm; **B** 10 μm; **C, E** 0.5 μm; **D, F** 200 nm.

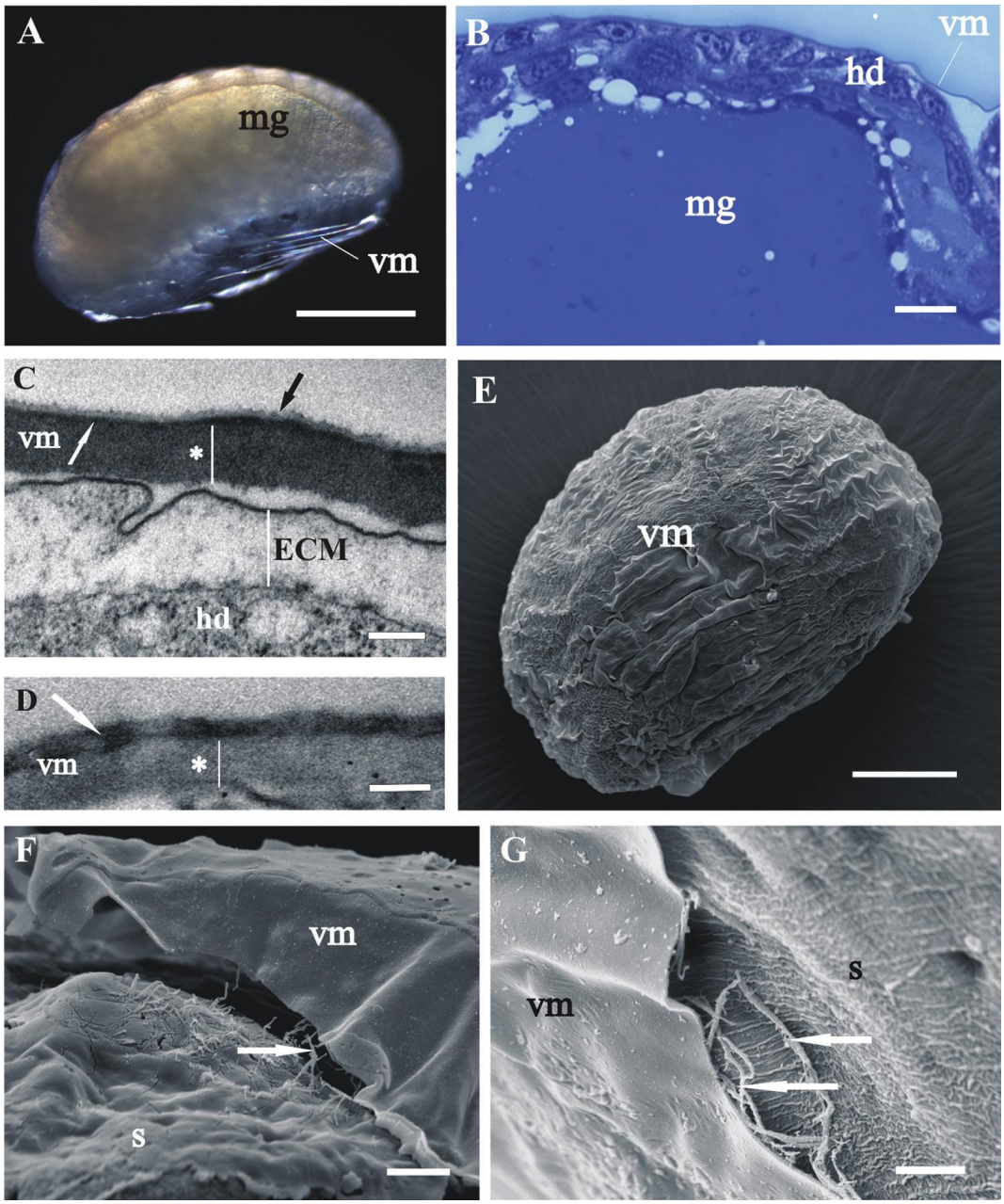

Figure 3. Structure of vitelline membrane (**vm**), covering *P. scaber* **A–D** and *P. dilatatus* **E–G** late-stage embryo. **A** Ventrally bent late-stage embryo, yolk is completely enclosed into the midgut glands (**mg**). **B** Semithin section of the embryo peripheral region. Vitelline membrane is slightly detached from the hypodermis (**hd**). **C, D** TEM micrographs of the vitelline membrane in osmicated specimen **C** and in non-osmicated specimen **D** Main proximal homogenous layer (*), thin middle electron dense layer (white arrow) and superficial corrugated lucent layer (black arrow). Hypodermis is covered with an extracellular matrix (ECM). **E** SEM micrograph of the late-stage embryo surrounded by vitelline membrane. **F, G** SEM micrographs of the late-stage embryo surface area. The vitelline membrane is artificially slit and fibers (arrows) between the outer embryo surface, covered with an extracellular matrix (s), and the vitelline membrane are exposed. Bars: **A** 500 μm; **B, F** 10 μm; **C, D** 200 nm; **E** 200 μm; **G** 5 μm.

stage, in which the overall ultrastructural architecture of the cuticle is similar to the adult crustacean cuticle and first morphological evidence of cuticle renewal is evident. Cuticle is from 2 to 3 μm thick, which is significantly thinner than in adults. It is composed of three layers, the outermost thin electron dense epicuticle, the middle exocuticle and the innermost endocuticle (Figs 4C, D, E). Cuticular scales are fully elaborated (Fig. 4B, D). Several transverse sections of completely structured sensilla are observed in the hypodermis (Fig. 4F). Dendritic outer segments and enveloping cells are clearly differentiated. The characteristic pattern of chitin-protein fibers arrangement in the exocuticle is resolved in some regions and hardly discernible in other regions of the same specimen. The endocuticle is subdivided in several electron dense sublayers alternating with electron lucent sublayers (Figs 4C, D, E). In some regions pore canals are visible running through the endocuticle, consisting of electron lucent central part and electron dense margins (Fig. 4C). Several morphological features in prehatching late-stage embryo show the exoskeletal cuticle renewal already in this stage of development: partly disintegrated proximal portion of endocuticle in some regions (Fig. 4C); cuticle detachment from the hypodermis (Figs 4B, C, D, E); rough apical plasma membranes of hypodermal cells and irregularly arranged electron dense particles on their outer surface (Figs 4C, E).

Next, we present evidence of cuticle renewal in different stages of marsupial mancas, namely: (I) early-stage marsupial manca, immediately after hatching (Fig. 5A); (II) mid-stage marsupial manca (Fig. 5B) and (III) late-stage marsupial manca, just prior to release from the marsupium (Fig. 5C). In the marsupial mancas the cuticle has similar architecture as the cuticle of prehatching embryo (Figs 5D–I). The main difference is the more pronounced characteristic chitin-protein pattern of exocuticle with a dense distal layer in marsupial mancas in comparison to embryo (Figs 5G, I). The micrograph of the late-stage marsupial manca cuticle displays well-formed pattern of exocuticle, resembling the adult helicoidal exocuticle structure and with a dense distal layer (Fig. 5I). The endocuticle of marsupial mancas is perforated by pore canals. The thickness of the marsupial manca cuticles is up to 3 μm. Morphological characteristics of cuticle renewal are observed in all three stages of marsupial mancas (Figs 5G-K). Apolysis, detachment of the old cuticle from the hypodermis is clearly visible in several stages (Figs 5F, H, I). The detached cuticle is partly degraded and much thinner in certain regions of the same specimen. The ecdysal space between the detached cuticle and the newly forming cuticle is also well evident and it contains homogenous electron dense material in some specimens (Fig. 5H), while in others it appears devoid of electron dense material (Fig. 5I). The newly assembling cuticle, covering hypodermal cells, consists of two layers, a thin electron dense external layer - epicuticle and an electron lucent procuticle (Figs 5H, I, K). In some regions of late-stage marsupial manca the procuticle appears homogenous (Fig. 5I), while in other regions helicoidal chitin-protein fibers arrangement is clearly discernible (Fig. 5K). The surface of the new cuticle is slightly (Fig. 5H) or highly wrinkled (Fig. 5I). Protrusions with electron dense tips are formed on apical surfaces of hypodermal cells. These characteristic features of cuticle

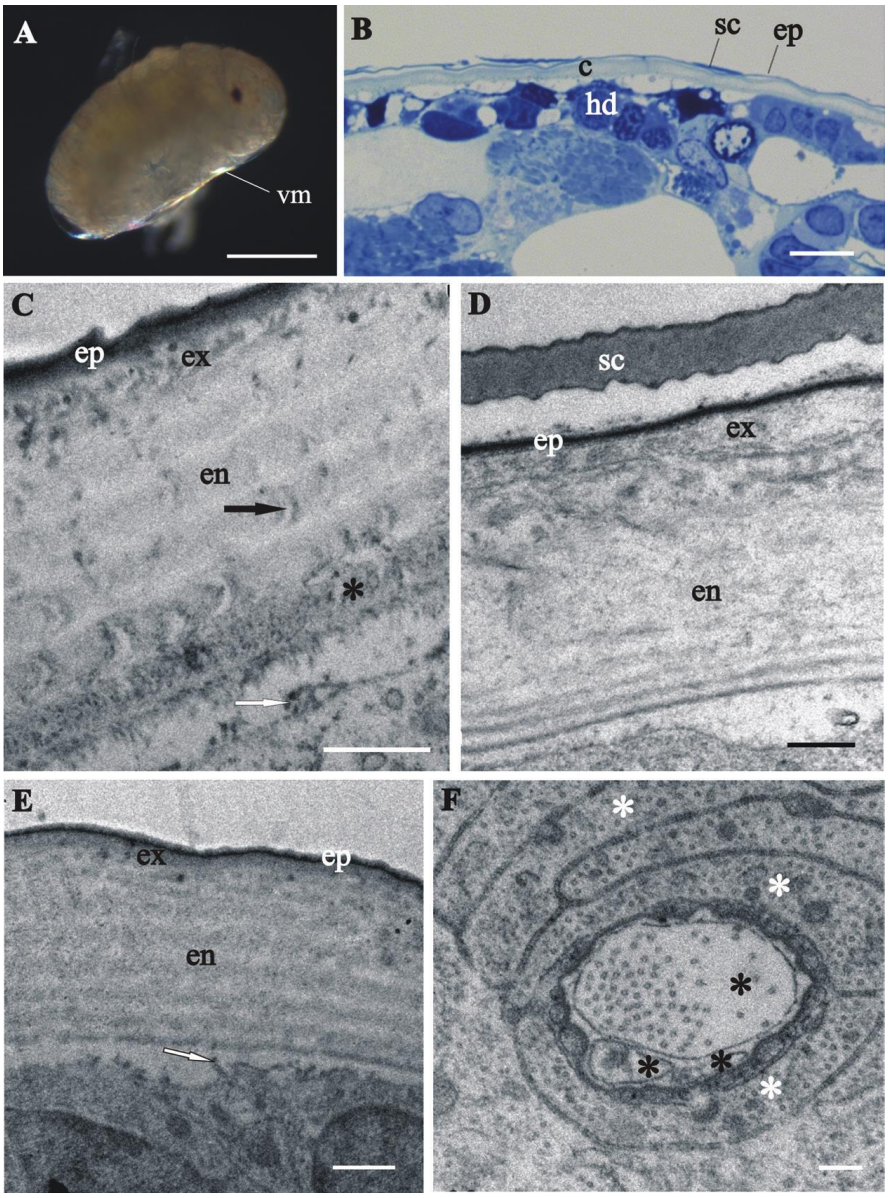

Figure 4. Cuticle structure and renewal in *P. scaber* prehatching late-stage embryo. **A** Swelled embryo inside the vitelline membrane (**vm**), prior to hatching. **B** Semithin section of the embryo peripheral region. The vitelline membrane is artificially removed. Clearly discernible exoskeletal cuticle (**c**), detached from the underlying hypodermis (**hd**). **C, D, E** TEM micrographs of exoskeletal cuticle in different regions of the same specimen, composed of three principal layers: the outermost thin electron dense epicuticle (**ep**), the middle exocuticle (**ex**) and the innermost endocuticle with several sublayers (**en**). The micrographs show features of cuticle renewal: cuticle detachment from the hypodermis, partial disintegration of proximal portion of endocuticle (*) and irregularly arranged electron dense particles on outer apical plasma membrane surface (white arrows). Pore canals (black arrow) in the endocuticle consist of electron lucent central part and electron dense margins **C**. Cuticular scales (**sc**) are fully elaborated and the exocuticle has the characteristic pattern of chitin-protein fibers arrangement **D**. Exocuticle is hardly discernible **E**. **F** TEM micrograph of completely structured sensillum transverse section in the hypodermis. Dendritic outer segments (*) and enveloping cells (white *). Bars: **A** 500 μm; **B** 10 μm; **C, E** 1 μm; **D** 0.5 μm; **F** 200 nm.

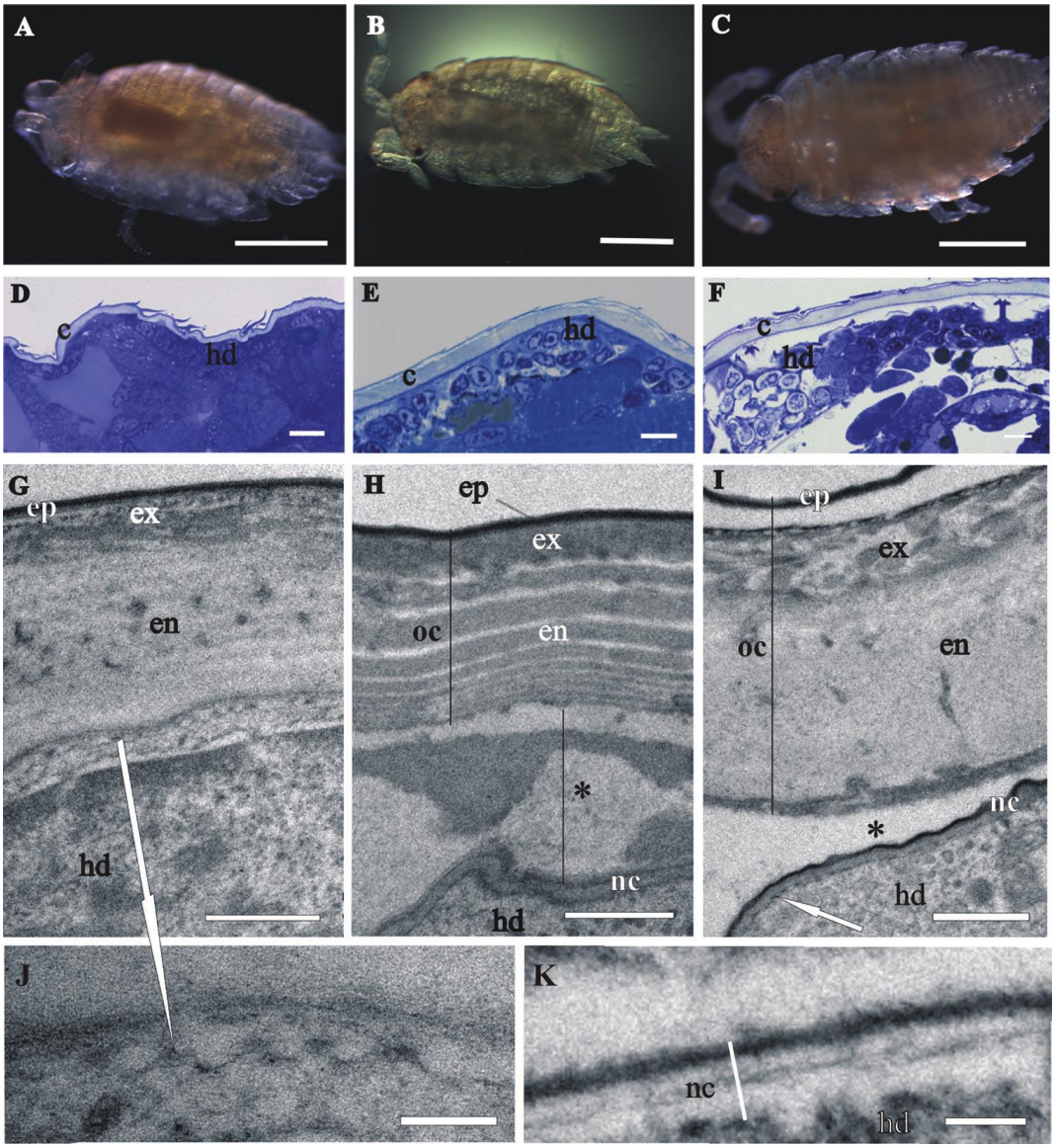

Figure 5. Cuticle structure and renewal in *P. scaber* marsupial mancas. **A** The early-stage marsupial manca, immediately after hatching **B** The mid-stage marsupial manca **C** The late-stage marsupial manca, just prior to release from the marsupium **D–F** Semithin sections of the manca peripheral region in the early-stage marsupial manca **D** in the mid-stage marsupial manca **E** and in the late-stage marsupial manca **F**. Cuticle (**c**), overlying the hypodermis (**hd**), becomes progressively more similar to adult cuticle. **G–K** TEM micrographs of exoskeletal cuticle in the early-stage marsupial manca **G, J** in the mid-stage marsupial manca **H** and in the late-stage marsupial manca **I, K** Three main layers are distinguished: epicuticle (**ep**), exocuticle (**ex**) and endocuticle (**en**). The micrographs show morphological characteristics of cuticle renewal: detachment of the old cuticle (**oc**) from the hypodermis, ecdysal space (*) between the detached cuticle and the newly forming cuticle (**nc**) and partial degradation of the old cuticle **H, I** protrusions with electron dense tips (white arrows) on apical surfaces of hypodermal cells **G, I, J**. The new cuticle consists of two layers, external electron dense epicuticle and internal electron lucent procuticle **H, I, K**. Helicoidal chitin-protein fibers arrangement is discernible in some regions of late-stage marsupial manca **K**. Bars: **A–C** 500 μm; **D–F** 10 μm; **G–I** 1 μm; **J, K** 200 nm.

synthesis are clearly visible underneath the assembling cuticle in all stages of marsupial mancas (Figs 5G, I, J).

The results of histochemical reaction with Alizarin red S for calcified tissues localization indicate that the cuticle of marsupial mancas is not strongly mineralized (Fig. 6A) in comparison to adult cuticle (Fig. 6B). The ventral calcium deposits, characteristic for adult premolt animals, were not observed in molting marsupial mancas.

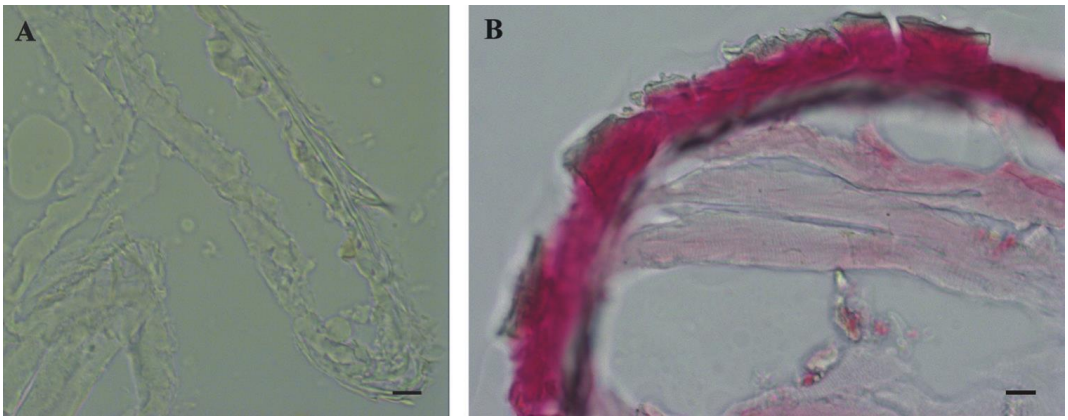

Figure 6. Histochemical reaction for calcium – Alizarin red S. **A** No reaction in *P. scaber* late marsupial manca **B** Positive control (red-pink) in adult *P. scaber* cuticle. Bars: 10 μm.

Discussion

The structure of egg envelopes and the ultrastructural architecture of the exoskeletal cuticle in *Porcellio* embryos and marsupial mancas share some common features with those described in other arthropods, but there are also some significant differences.

In comparison to egg envelopes of *Drosophila* eggs from ovaries, described by Margaritis et al. (1980), the egg envelopes of *Porcellio* embryos are thinner and have considerably less complex ultrastructure. Chorion of *Porcellio* is approximately two times thinner than chorion of *Drosophila.* The chorion of *Drosophila* eggs is differentiated into several layers, a thin innermost chorionic layer, a complex fenestrated endochorion and a fibrous exochorion, while *Porcellio* chorion in embryos consists of a single homogenous layer. Electron lucent "lacunae" observed in the *Porcellio* chorion resemble the cavities in the inner part of the *Drosophila* chorion. These complex cavities are thought to be air-filled in laid eggs and involved in respiration (Margaritis et al. 1980). Study of embryo tolerance to physiological stresses in terrestrial isopod *Armadillidium vulgare* shows that the chorion has low permeability to water and solutes and contributes to the very high tolerance to osmotic stress of early-stage embryo (Surbida and Wright 2001). The vitelline membrane of *Porcellio* embryos also appears thinner, but has a similar ultrastructure as *Drosophila* eggs vitelline membrane. Our results indicate that the osmiophilic main proximal layer and the thin superficial layer of *Porcellio* vitelline membrane differ in composition from the middle electron dense layer. The thin superficial layer may correspond to a similar thin layer above vitelline

membrane in *Drosophila* egg (Margaritis et al. 1980). The authors presume that this layer in *Drosophila* consists of wax, functioning to reduce water loss. This is not expected for *Porcellio* embryos since they are less vulnerable to desiccation due to their development in aqueous environment. The comparison of egg envelopes ultrastructure in *Porcellio* embryos and in *Drosophila* eggs reveals several differences. We consider these dissimilarities the consequence of different environments of embryonic development in *Porcellio* embryos which develop in a protected fluid-filled maternal brood pouch and in *Drosophila* embryos which are directly exposed to external environment during development. Here we also report on a network of fibers between embryo surface and vitelline membrane in late-stage embryo. We presume that these fibers function as connective elements between embryo and envelope, but no comparative data on vitelline membrane attachment were found in the literature.

We show here that the late embryo is already covered with a homogenous extracellular matrix, possibly first cuticle. In the prehatching late-stage embryo of *P. scaber* the exoskeletal cuticle has already main features of the adult crustacean cuticle, but minor differences are evident. It is composed of three principal layers - epicuticle, exocuticle and endocuticle. Endocuticle shows the typical arrangement of chitin lamellae in sublayers which are not so distinct as in adult cuticle. The characteristic pattern of chitin-protein fibers arrangement in the exocuticle of adults is not discernible in exocuticle of the prehatching late-stage embryo. A distal layer, similar to the dense distal exocuticular layer, described in adult isopods (Hild et al. 2009, Huber and Ziegler 2011, Seidl and Ziegler 2011), is not observed in the cuticle of prehatching embryo. Sensilla in hypodermis are already very elaborated and have similar ultrastructure as tricorn sensilla described in adult *P. scaber* (Ziegler and Altner 1995). Previous studies of cuticle differentiation during embryonic development of *Drosophila melanogaster* and *Parhyale hawaiensis* show that cuticle ultrastructure in last stage embryo likewise resembles adult cuticle ultrastructure, with typical helicoidal arrangement of chitin lamellae in procuticle and several-layered epicuticle (Locke 2001, Moussian et al. 2006, Havemann et al. 2008, Moussian 2010). Our observations of hypodermal cells in the prehatching late-stage embryo are in agreement with those described in *Drosophila*, as they are both flattened, with centrally placed nucleus, scarce organelles and connected by septate junctions (Moussian et al. 2006). During development of *P. scaber* marsupial mancas cuticle becomes progressively more similar to adult cuticle, particularly regarding the characteristic pattern of chitin-protein fibers in exo- and endocuticle, which is more and more explicit. In the distal portion of the exocuticle an electron dense layer is clearly resolved. It could correspond to the dense distal exocuticular layer, described in several adult isopods (Hild et al. 2009, Huber and Ziegler 2011, Seidl and Ziegler 2011). Several authors report on possibility of exoskeleton calcification in marsupial mancas. Inferred only by increased total calcium concentration of *Armadillidium vulgare* late-stage marsupial manca, Ouyang and Wright (2005) suggest that cuticle calcification starts in this stage. Surbida and Wright (2001) presume that wide osmotic tolerance of *A. vulgare* marsupial mancas is a consequence of calcification of their cuticle. Havemann et al. (2008) report on cuticle calcification after hatching of amphipod

Parhyale hawaiensis embryo, but it is not known which larval stage was observed. Our research, using histochemical approach to localize calcified tissue, indicates that the exoskeletal cuticle of *P. scaber* marsupial mancas is not strongly, if at all mineralized. It could be possible that amorphous calcium carbonate was dissolved during preparation, since it has relatively high solubility.

Next, the issue of cuticle renewal during intramarsupial development was addressed in this study. Partly disintegrated proximal endocuticle and cuticle detachment from the underlying hypodermis indicate that cuticle renewal takes place already in the prehatching embryonic stage. Prehatching embryo is thus the earliest stage of *P. scaber* development, where renewal of exoskeleton, i.e. initiation of molting, was observed so far. These results are in agreement with the previous observation of apolysis on appendage tips in late-stage embryo of *P. scaber* (Milatovič et al. 2010). Apolysis and cuticle disintegration were not observed in the studies of cuticle structure during embryonic development in insect *Drosophila melanogaster* and amphipod crustacean *Parhyale hawaiensis* (Locke 2001, Moussian et al. 2006, Havemann et al. 2008, Moussian 2010). We observed that the old cuticle is detached from the hypodermis and the new cuticle is produced in marsupial mancas of different stages, which is a sign of premolt. Several similarities and differences with respect to molting process in adult isopods are described. Similarities in apical protrusions of hypodermal cells during cuticle synthesis, and appearance of ecdysal space are very explicit. Regarding synthesis of newly assembling cuticle prior to ecdysis we show that preecdysal cuticle in mancas has mostly homogenous procuticle, with no distinct chitin-protein arrangement, although in certain regions a helicoidal arrangement of chitin-protein fibers is evident. In adult *P. scaber* it is reported that several exocuticular lamellae are deposited in premolt stage (Ziegler 1997). Advanced stages of new cuticle formation in marsupial mancas need to be investigated in further research. Molting in two phases is typical in adult terrestrial isopods, while this is still not confirmed for developing marsupial mancas. In adult isopods some other morphological changes accompany molting process, particularly concerning calcium dynamics. In adult premolt stage ecdysal space in-between the old and new cuticle contains calcium storage granules (Ziegler 1994, Štrus and Blejec 2001). In mancas no similar granules were observed in the ecdysal space. In adults appearance of calcium deposits on the first four anterior sternites is a clear indication of the premolt stage (Steel 1982, Zidar et al. 1998). In our study calcium deposits in molting marsupial stages were not observed, indicating differences in calcium dynamics compared to adults. There are no data on calcium dynamics during marsupial development in terrestrial isopods.

Ultrastructural research of isopod egg envelopes and cuticle structure during ontogenetic development in a fluid-filled marsupium is a valuable approach to get insight into their differentiation and function and contribute to comparative analysis of ontogenetic development in different arthropods. Evaluation of the data on the newly forming cuticle structure and composition obtained in sequential phases of the ontogenetic development is important to get insight into the mechanisms of mineralized biological matrix assembly.

Conclusions

During marsupial development of *Porcellio scaber* embryos and marsupial mancas in different stages are coated by different protective envelopes (Fig. 7).

Egg envelopes of isopod crustacean *Porcellio scaber* embryos are thinner and structurally less complex in comparison to egg envelopes of insect *Drosophila melanogaster*. These are expected differences due to different embryonic developmental strategies of these arthropods. Similarities in egg envelopes of these two species appear particularly in their inner egg envelope, the vitelline membrane.

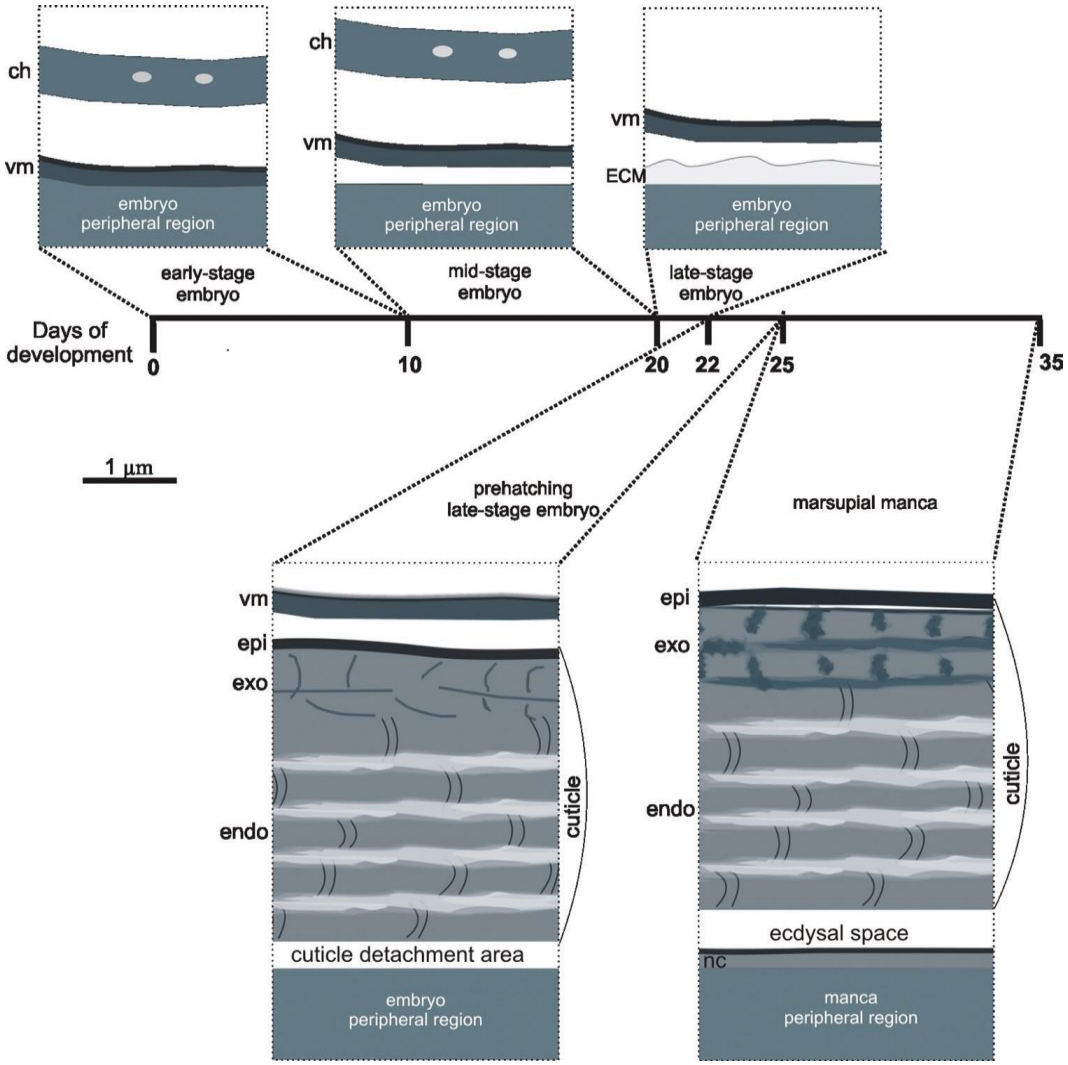

Figure 7. Schematic representation of different protective envelopes, coating *Porcellio scaber* embryos and marsupial mancas during development (lasting 35 days), namely egg envelopes (chorion and vitelline membrane) and exoskeletal cuticle. During growth of embryos and mancas egg envelopes are shed and cuticle is renewed. **ch –** chorion; **vm –** vitelline membrane; **ECM –** extracellular matrix; **epi –** epicuticle; **exo –** exocuticle; **endo –** endocuticle; **nc –** newly assembling cuticle.

Exoskeletal cuticles of *Porcellio scaber* prehatching late-stage embryo and marsupial mancas have already some features of the adult crustacean cuticle, but are significantly thinner. Three principal layers are distinguished, the outermost epicuticle, the middle exocuticle and the innermost endocuticle. Characteristic chitin-protein patterns of adult cuticle, particularly regarding the exocuticle, are not very distinct in prehatching late-stage embryo and become progressively more explicit in marsupial mancas. Cuticular scales and sensilla are fully elaborated already in prehatching late-stage embryo. In the distal portion of the exocuticle a dense layer is observed in marsupial mancas, which could correspond to the dense distal exocuticular layer of adult cuticle. Marsupial manca cuticle is not strongly calcified.

Cuticle renewal takes place already in prehatching late-stage embryo, where detachment of cuticle from hypodermis and partial disintegration of proximal endocuticle occur. Old cuticle detachment and new cuticle assembly appear in marsupial mancas of several stages. Morphological changes, related to calcium storage during molt cycle in adult isopods, were not observed in premolt marsupial stages.

Acknowledgements

We would like to thank Jožica Murko Bulić, Miloš Vittori and dr. Rok Kostanjšek for their laboratory assistance and all help and suggestions. This work was financed by the Slovenian Research Agency (ARRS), grand No. P1-0184 and 1000-11-310087.

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

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

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Acta Biologica Slovenica, 2013, 56(2): 51-62

Trdnost eksoskeletne kutikule rakov je posledica sklerotizacije in kalcifikacije organskega matriksa. Pomemben proces pri nastajanju nove kutikule je torej tudi kalcifikacija, nalaganje kalcijevih mineralov v organski matriks. Popolnoma oblikovana kutikula odraslih rakov vsebuje kalcijev karbonat v kristalni obliki (kalcit in Mg-kalcit), amorfni kalcijev karbonat (ACC) in amorfni kalcijev fosfat (ACP). V tej raziskavi smo na histokemijskem nivoju ugotavljali, ali je eksoskeletna kutikula marzupijskih mank že kalcificirana. V ta namen smo uporabili histokemijsko tehniko za lokalizacijo kalcificiranega tkiva z barvilom alizarin rdeče S (ARS), ki omogoča preprost in hiter pregled večjega števila vzorcev in je osnova za nadaljnje bolj zahtevne in natančne tehnike. Mineralizacija hitinskega matriksa pri larvah mankah še ni poznana. Nekaj podatkov je na voljo le za manke po sprostitvi v zunanje okolje (postmarzupijske manke), pri katerih so z analizno elektronsko mikroskopsko tehniko EDXS ugotovili prisotnost kalcija v kutikuli (Hadley in Hendricks, 1987), medtem ko kalcifikacija kutikule pri marzupijskih mankah ni znana. Glede na to, da je v literaturi uporabljenih več modifikacij histokemijske analize z barvilom ARS, ki se primarno uporablja v histologiji vretenčarjev, smo na vzorcih mank in odraslih rakov enakonožcev *Porcellio scaber* izvedli in ovrednotili petnajst različnih postopkov te metode. Izvedli smo pet različnih načinov fiksacije tkiva: (a1) fiksacija v fiksativu Carnoy; (a2) fiksacija v 3.7% raztopini formaldehida v 0.1 M kakodilatnem pufru (pH 7.2); (a3) fiksacija v 70% etanol; (b) fiksacija v 3.7% raztopini formaldehida v 0.1 M kakodilatnem pufru (pH 7.2) in zamrzovanje; (c) fiksacija z zamrzovanjem. Barvali smo s tremi različnimi raztopinami barvila: ARS 1 (pH 9), ARS 2 (pH 6.4) ali ARS 3 (pH 4.8). Zamrzovanje in barvanje kriostatskih rezin smo vključili z namenom, da bi se med postopkom priprave tkiva čimbolj izognili vodnim raztopinam, v katerih so amorfne oblike kalcijevih soli dobro topne. Tako pri odraslih kot pri mankah se je eksoskeletna kutikula izrazito diferencialno obarvala v primeru kratkotrajne fiksacije z nevtralno raztopino formaldehida, ki ji je sledilo barvanje parafinskih rezin z eno izmed raztopin ARS: ARS 1 (pH 9), ARS 2 (pH 6.4) ali ARS 3 (pH 4.8). Jasno lokalizacijo smo dosegli tudi z barvanjem zamrznjenih rezin vzorcev, fiksiranih v formaldehidu in barvanih z raztopino ARS 1 (pH 9). Pokazali smo, da alkoholni fiksativi (fiksativ Carnoy in 70% etanol) niso primerni za diferencialno lokalizacijo kalcificiranega tkiva, saj obarvanje rezin ni bilo jasno specifično. V primerih zamrzovanja po kemijski fiksaciji in barvanja s kislimi

barvili ter v primerih vseh treh barvanj samo zamrznjenih vzorcev smo dobili nespecifično obarvanje tkiv ali pa so nastale druge pomanjkljivosti, kot so difuzijski artefakti ali neenakomerno barvanje. Rezultati kažejo, da je eksoskeletna kutikula pri mokricah *P. scaber* znatno kalcificirana že v postembrionalnem razvoju ličink mank v valilniku. Kalcificiranost eksoskeleta je najverjetneje pomembna za zaščito živali in za gibanje mank, ki smo ga opazili pred sprostitvijo iz valilnika. Pričakujemo, da bodo postopki, ki jih predlagamo, uporabni tudi pri študijah kalcifikacije drugih hitinskih matriksov.

ABS ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2013 Vol. 56, Št. 2: 51–62

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

Izvleček: Eksoskeletna kutikula rakov je hitinski matriks na apikalni strani epidermisa. Trdnost kutikule je posledica sklerotizacije in kalcifikacije organskega matriksa. Embriji kopenskega raka enakonožca *Porcellio scaber* se razvijajo v vrečastem valilniku samice, marzupiju, kjer po izleganju nadaljujejo svoj razvoj približno en teden tudi ličinke manke. S histokemijsko metodo smo ugotavljali, ali je eksoskeletna kutikula marzupijskih mank že kalcificirana. Na vzorcih mank in odraslih rakov *P. scaber* smo ovrednotili petnajst različnih postopkov histokemijske analize z barvilom alizarin rdeče S (ARS), ki se uporabljajo za lokalizacijo kalcificiranega tkiva v histologiji vretenčarjev. Eksoskeletna kutikula se je izrazito diferencialno obarvala v primeru fiksacije z nevtralno raztopino formaldehida, ki ji je sledilo

barvanje parafinskih rezin z eno izmed raztopin ARS: ARS 1 (pH 9), ARS 2 (pH 6.4) ali ARS 3 (pH 4.8). Jasno lokalizacijo smo dosegli tudi z barvanjem zamrznjenih rezin vzorcev, fiksiranih v formaldehidu in barvanih z raztopino ARS 1 (pH 9). Rezultati kažejo na znatno kalcifikacijo eksoskeletne kutikule že v postembrionalnem razvoju mank *P. scaber* v marzupiju. Trdnost eksoskeleta je najverjetneje pomembna tudi za gibanje mank, ki smo ga opazili pred sprostitvijo iz valilnika. Pričakujemo, da bodo postopki, ki jih predlagamo, uporabni tudi za histokemijska proučevanja kalcifikacije drugih hitinskih matriksov.

Ključne besede: kalcifikacija, histokemija, razvoj ličink, kopenski raki enakonožci, Crustacea

Introduction

Mineralized organic matrices constitute many morphologically and functionally diverse structures in different organisms ranging from bacteria to humans. Their unique feature is a prominent mineral component, which is closely connected with the organic matrix. Mineralization increases matrix strength and hardness that provides protection against environmental pressures and support for muscle attachment. A common type of mineralization in living organisms is calcification, the deposition of different calcium minerals in the organic matrices (Bonucci 2007). Well known examples of calcified organic matrices in vertebrates are bones and teeth, whose basic organic component is collagen. Exoskeleton of crustaceans or exoskeletal cuticle is a representative example of a calcified matrix based on chitinous organic scaffold.

The exoskeletal cuticle is a complex hierarchically structured extracellular matrix, consisting of the polysaccharide chitin, proteins, lipids and also minerals. It is produced by a single-layered epidermis during embryonic development and it is renewed periodically during molting in adults. The ultrastructure of exoskeletal cuticle in adult isopod *Porcellio scaber* has been described in detail (Ziegler 1997, Hild et al. 2008, Seidl and Ziegler 2012). It comprises the outermost epicuticle, exocuticle and the inner endocuticle. Thin epicuticle is composed mainly of lipoproteins and consists of thinner 5-layered outer epicuticle and thicker inner epicuticle. Exocuticle and endocuticle are calcified and comprise sublayers of chitin-protein fibers arranged in a characteristic helicoidal pattern (Fig. 1).

Cuticle in adults is calcified due to deposition of crystalline calcium carbonate (calcite), amorphous calcium carbonate (ACC) and amorphous calcium phosphate (ACP) (Ziegler 1994, Ziegler 1997, Becker et al. 2005, Luquet 2012). Recent studies have shown defined spatial distribution of both polymorphs of calcium carbonate in different layers of adult isopod cuticle (Hild et al. 2008, Hild et al. 2009, Neues et al. 2011, Seidl et al. 2011). In *P. scaber* the exocuticle contains both calcite and ACC, whereas the endocuticle is calcified only by ACC (Hild et al. 2008). There are a few data on the structure and composition of the exoskeletal cuticle in embryonic and larval stages in crustaceans. The central focus of this study is to show at the histochemical level whether the larval cuticle in *Porcellio scaber* is calcified or not.

The specimens of *Porcellio scaber* belong to a group of terrestrial isopod crustaceans (Oniscidea). The embryonic development takes place in the fluid-filled brood pouch (marsupium) on the ventral side of female body, that has likely been of a great adaptive significance in the colonization of land by crustacean species (Hornung 2011, Warburg 2011). Intramarsupial development of *P. scaber*, from released fertilized eggs to embryos and marsupial larvae mancae, lasts approximately 35 days in laboratory conditions and it was described morphologically with twenty progressive stages (Wolff 2009, Milatovič et al. 2010). After hatching of embryo from two egg envelopes (chorion and vitelline membrane), larva termed manca stays in the marsupium for a week and is subsequently released to the external environment (Supplementary fig. 1, supplementary figures are available in online edition).

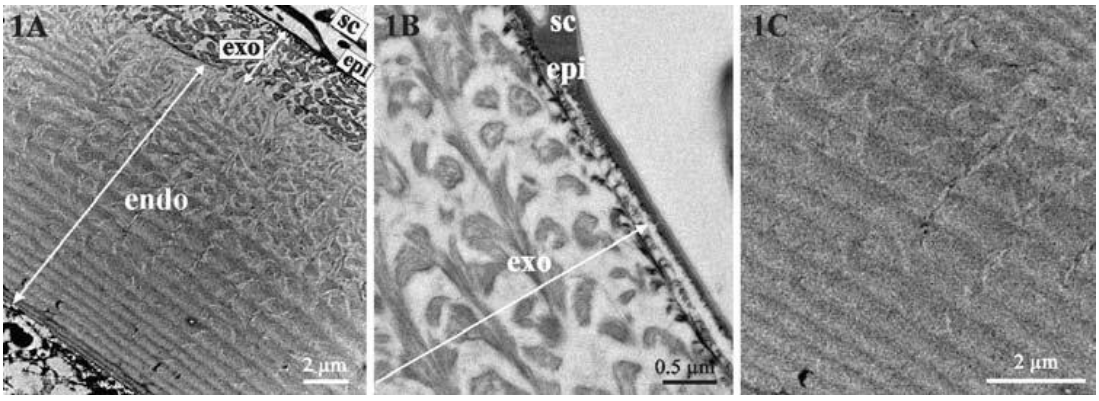

Figure 1: Ultrastructure of the exoskeletal cuticle in adult *P. scaber*. A – Cuticle is organized in distinct horizontal layers: epicuticle (epi), exocuticle (exo) and endocuticle (endo). B – Epicuticle (epi) is a thin and electron dense layer with prominent scales (sc). Exocuticle (exo) comprises chitin-protein fibers, arranged in a characteristic pattern. C – Endocuticle comprises lamellar chitin-protein sublayers.

Slika 1: Ultrastruktura eksoskeletne kutikule odraslega raka *P. scaber.* A – Kutikulo sestavljajo različni sloji: epikutikula (epi), eksokutikula (exo) in endokutikula (endo), kot si sledijo od zunanjega proti notranjemu delu. B – Epikutikula (epi) je tanek, elektronsko gost sloj z izrazitimi luskami na površini (sc). Eksokutikula (exo) vsebuje hitinsko-proteinska vlakna, ki so urejena v značilen vzorec. C – Endokutikula vsebuje lamelarne podsloje hitinsko-proteinskih vlaken.

Our previous study shows that the cuticle in *P. scaber* marsupial mancae exhibits main ultrastructural features of the adult crustacean cuticle (Mrak et al. 2012). Cuticle of marsupial mancae is composed of three layers, the outermost thin electron dense epicuticle, the middle exocuticle and the innermost endocuticle. The characteristic pattern of chitin-protein fiber arrangement in the exocuticle is already present and sublayers are evident in the endocuticle (Fig. 2). The thickness of cuticle is up to 3 µm. Some morphological features suggesting cuticle renewal are observed occasionally in marsupial mancae, such as cuticle detachment from the epidermis, partly disintegrated inner portion of the endocuticle, assembling of the new cuticle and apical protrusions of epidermal cells with electron dense tips (Fig. 2B).

The data on mineralization of chitin matrix in mancae larvae is very limited. Mancae, released from the marsupium (postmarsupial mancae), were investigated in this respect by Hadley and Hendricks (1987) using energy dispersive X-ray spectroscopy (EDS) in the isopod *Porcellionides pruinosus*. They detected calcium in the cuticle of mancae already released to the external environment, but in much lower quantities compared to the adult cuticle levels. Concerning marsupial mancae, exoskeleton calcification is still an open question.

Localization and characterization of calcified tissues can be performed by different methods, each of them focused to address specific questions regarding tissue composition and structure. Identification of inorganic components can be achieved using different biophysical and morphological methods (Bonucci 2007). The advantage of histochemical techniques is simple and quick performance that is important when screening of many samples is needed to gain preliminary information about the presence of mineralized tissue. On the basis of these results selected samples can be further analysed in detail with advanced and highly demanding biophysical techniques, like: X-ray, neutron and electron diffraction for studying the structure of crystals; energy dispersive X-ray elemental analysis (EDS) and electron energy-loss spectroscopy (EELS) for analysing the presence of specific elements and their distribution in the tissue; infrared and Raman spectroscopy for revealing details about molecular structure and especially mineral forms in the tissue. A commonly used histochemical method to demonstrate calcified tissue, applied also in our study, is alizarin red S (ARS) staining. According to Virtanen and Isotupa (1980) and Lievremont et al. (1982), alizarin red S molecules react with calcium ions via its sulfonate and hydroxyl groups, forming brick-red

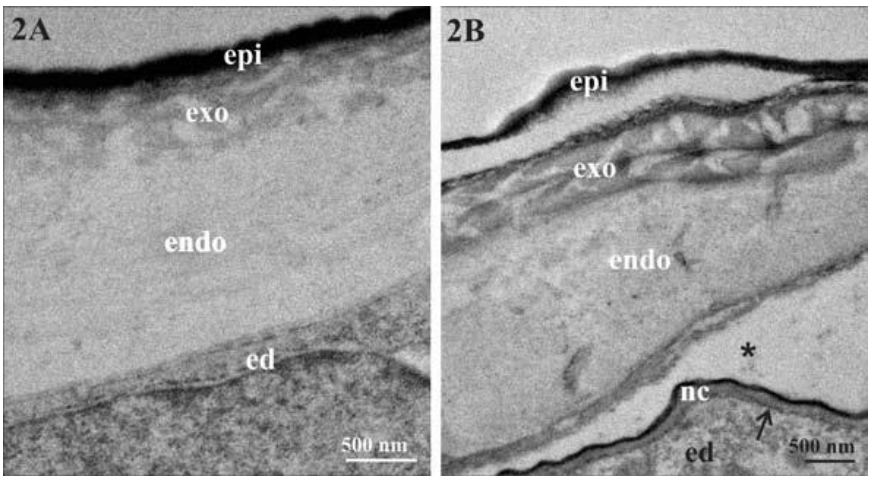

Figure 2: Ultrastructure of *P. scaber* marsupial manca cuticle. Differentiation in epicuticle (epi), exocuticle (exo) and endocuticle (endo) is evident. The micrograph B shows some features of cuticle renewal: cuticle detachment from the epidermis, ecdysal space (*) between the old and the new cuticle, partial disintegration of inner portion of endocuticle, newly assembling cuticle (nc) and protrusions with electron dense tips (arrow) on apical plasma membrane of epidermal cell (ed).

Slika 2: Ultrastruktura kutikule pri marzupijskih mankah raka *P. scaber*. Diferenciacija v epikutikulo (epi), eksokutikulo (exo) in endokutikulo (endo). Mikrografija B prikazuje nekatere značilnosti obnavljanja kutikule, kot so: odstopanje kutikule od epidermisa, levitveni prostor (*) med staro in novo kutikulo, delna razgradnja notranjega dela endokutikule, nastajanje nove kutikule (nc) in izrastki z elektronsko gostimi konicami (puščica) na apikalni plazmalemi epidermalnih celic (ed).

precipitates (ARS-calcium salts, complexes and chelates). Other cations (like magnesium, barium, copper, zinc, iron, aluminium) react with ARS as well, but normally only calcium is present in biological tissues in sufficient quantities for demonstration (McGee-Russell 1958, Puchtler et al. 1969, Lievremont et al. 1982). ARS is also an anionic dye and as such causes non-specific pink background staining. According to the literature, that reports several modifications in procedure of the staining technique, the histochemical result is greatly influenced by the fixation method and pH of staining solution. Fixation method has a significant effect on preservation of calcium in tissue, while pH of staining solutions effects staining sensitivity and staining specificity. As this method has relatively low sensitivity, small differences in calcium concentrations can not be distinguished and low calcium concentrations can not be detected.

This study, based on alizarin red S staining, a histochemical approach for calcified tissue localization, was performed to show whether cuticle is calcified already during intramarsupial larval development in isopod crustacean *Porcellio scaber*. Considering many modifications of this method, particularly regarding fixation and staining pH, we have evaluated several procedures for estimating the presence of cuticle calcification in *P. scaber* marsupial mancae. This method is expected to be useful also in the studies of calcification of other chitinous matrices.

Materials and methods

In this study the histochemical localization of calcified tissues by alizarin red S (ARS) was implemented to estimate the presence of exoskeleton calcification in marsupial mancae of the species *Porcellio scaber* Latreille, 1809 (Crustacea: Isopoda). Adult animals were maintained in a laboratory culture, in soil and leaf litter, at 25 °C, high relative humidity and a 12-h light/12-h dark cycle. Gravid females with mancae in the marsupium were selected from laboratory culture. Thirteen mancae were isolated from the marsupia, anesthetized and fixed. They were carefully perforated with a thin needle, enabling better infiltration of the fixative and/or the embedding medium. Adult animals that were without any signs of molting were used as positive controls for histochemical reaction. Animals were anesthetized, transversely cut in two pieces and fixed. Subsequently they were always processed simultaneously with the samples of marsupial mancae in all procedures applied. Negative controls of histochemical

reaction were the sections of adult and mancae samples, preincubated in decalcification solution (10% ethylenediamine-tetracetic acid disodium salt (EDTA), pH 7.4, 5 minutes) before ARS staining.

Fixation was performed in five ways (Table 1): (a) three different chemical fixations, (b) chemical fixation followed by freezing or (c) freezing. In the procedure (a) – chemical fixation, three different fixatives were used, recommended by several authors: (a1) Carnoy fixative (absolute ethanol-chloroform-glacial acetic acid, 6:3:1), (a2) 3.7% formaldehyde in 0.1 M cacodylate buffer (pH 7.2) or (a3) 70% ethanol. Formaldehyde is a widely recommended fixative, also for studies of calcium localization (McGee-Russell 1958, Bancroft and Gamble 2008, Kiernan 2008), although some authors prefer Carnoy fixative and describe formaldehyde as unsuitable fixative for studies of calcium deposits as it could remove some calcium during prolonged fixation (Puchtler et al. 1969, Presnell and Schreibmann 1997). Alcoholic fixatives are widely recommended for calcified tissue localization as they preserve calcium in tissue (Puchtler et al. 1969, Presnell and Schreibmann 1997, Bancroft and Gamble 2008, Kiernan 2008), though it is a poor fixative for preservation of tissue structure. After fixation, samples were dehydrated through an ascending series of ethanol and in xylene, infiltrated with paraffin wax at 60 °C overnight and embedded afterwards. Transversal sections (10 µm) were cut with a Leica RM2265 microtome, transferred to water on microscope slides and stretched and dryed on a hot plate.

Samples prepared for paraffin sectioning were exposed to aqueous solutions several times, which could affect the tissue by removing amorphous calcium. Several authors demonstrated high solubility of ACC in aqueous solutions (Brečević and Nielsen 1989, Gal et al. 1996, Meiron et al. 2011). To avoid calcium loss from the tissue, cryosectioning was performed, which minimizes exposure to aqueous solutions. Specimens destined for cryosectioning were either fixed in 3.7% formaldehyde in 0.1 M cacodylate buffer (pH 7.2) and frozen afterwards (procedure b) or directly frozen without any pretreatment (procedure c). They were embedded in tissue freezing medium (Jung). Sections (10 µm) were cut with a Leica CM1850 cryostat at –20 °C. Cryosections were transferred directly to microscope slides and dryed at room temperature.

According to the literature, ARS staining is greatly influenced by pH of staining solution. Alkaline ARS solution liberates less calcium and has lower sensitivity but enables more precise localization of calcified tissue (Puchtler et al. 1969, Kiernan 2008). Acidity of ARS solution leads to releasing of more calcium ions from tissue and consequently calcium localization appears more dispersive and sensitivity for calcified tissue demonstration appears higher. Paraffin sections and cryosections were stained by three different staining solutions of ARS, that were recommended by several authors (McGee-Russell 1958, Puchtler et al. 1969, Presnell and Schreibmann 1997, Bancroft and Gamble 2008, Kiernan 2008). ARS solution 1 (purple-red coloured) was 0.5% solution in 0.2 M Trihydroxylmethyl aminomethane (Tris)-HCl buffer, pH 9. ARS solution 2 (dark red coloured) was 1% aqueous solution, adjusted to pH 6.4 with 10% NH_4OH. ARS solution 3 (brown-red coloured) was 1% aqueous solution, adjusted to pH 4.8 with 10% ammonium hydroxide (NH_4OH). Before staining, paraffin sections were deparaffinized and rehydrated in a descending series of ethanol to distilled water and after staining, they were dehydrated and mounted in synthetic resin Pertex. Cryosections were stained directly and after staining they were mounted in glicerol jelly. Staining duration was 20–30 seconds for paraffin sections and 10 seconds for cryosections, followed by a quick rinse in distilled water. The applied procedures are summarized in Table 1.

Sections were imaged by Zeiss AxioImager Z.1 light microscope, equipped with a HRc AxioCam camera and Axiovision software. Cryosections were inspected immediately after mounting. Intensity of the histochemical reaction with ARS was classified semiquantitatively in four categories: (i) no staining, (ii) light staining, (iii) moderate staining and (iv) intense staining.

Results

In this study the calcification of exoskeleton in marsupial mancae of *Porcellio scaber* Latreille, 1809 (Crustacea: Isopoda) was estimated at the

Table 1: Summary of the fixation and alizarin red S staining procedures, used in this study on *Porcellio scaber* marsupial mancae.

Tabela 1: Povzetek postopkov fiksacije in histokemijske lokalizacije z barvilom alizarin rdeče S, uporabljenih v tej študiji marzupijskih mank raka enakonožca *Porcellio scaber*.

	(a) chemical fixation		**(b) chemical fixation followed by freezing**	**(c) freezing at –20 °C**
(a1) Carnoy fixative	(a2) neutral buffered 3.7% formaldehyde	(a3) 70% ethanol	neutral buffered 3.7% formaldehyde	
	↓ dehydration ↓ paraffin embedding ↓		↓ tissue freezing medium embedding and freezing ↓	
	paraffin sectioning ↓ deparaffinization and rehydration			cryosectioning
	↓ staining in alizarin red S solution 1 (pH 9) or 2 (pH 6.4) or 3 (pH 4.8) ↓ quick rinse in distilled water			
	↓ dehydration ↓			↓
	mounting in resin			mounting in glicerol jelly

histochemical level by alizarin red S (ARS) method for calcified tissues localization. Several methods have been suggested for ARS staining and here we tested five different fixations in combination with three different staining solutions, altogether fifteen different procedures, on *P. scaber* marsupial mancae. We analysed the staining intensity of mancae cuticle in comparison to other tissues (background) and regarding positive and negative controls of histochemical reaction.

In Carnoy fixed specimens (procedure a1) no differential staining of the cuticle was obtained, neither in adults nor in mancae sections (Table 2, Supplementary fig. 2). ARS staining of Carnoy fixed specimens resulted in light red staining of all tissues with basic ARS solution (ARS 1). ARS 2 (pH 6.4) and ARS 3 (pH 4.8) solutions resulted in deeper red staining, displaying intensely stained cuticle and moderately stained other tissues, i.e. connective tissue and muscles (Table 2, Supplementary fig. 2).

ARS staining of neutral buffered formaldehyde fixed specimens (procedure a2) resulted in clearly differential staining of exoskeleton (Table 2, Supplementary fig. 3). The exoskeletal cuticle in adults and mancae was intensely red, while other tissues like glands and muscles were not stained. Negligible background was visible with acid ARS staining solution (ARS 3, pH 4.8). In specimens pretreated in decalcification solution (EDTA) for negative controls, the cuticle was not red stained with ARS and only in the case of ARS 2 and ARS 3 staining solutions negligible background was observed (Supplementary fig. 3).

The overall histological structure of specimens fixed in 70% ethanol (procedure a3) was not so well preserved in comparison to specimens fixed in formaldehyde (Supplementary fig. 4). We did not obtain any undoubtedly differential staining of the exoskeleton in ethanol fixed samples (Table 2, Supplementary fig. 4). In all mancae and adult sections cuticle and other tissues were stained nearly with the same intensity, except for the more intensely stained adult cuticle with basic ARS (ARS 1). In negative controls (pretreated in

Table 2: Summary of Alizarin red S staining results of *Porcellio scaber* marsupial mancae and adults: Specimens were fixed by five different methods: (a1) Carnoy fixative, (a2) 3.7% neutral buffered formaldehyde, (a3) 70% ethanol, (b) 3.7% neutral buffered formaldehyde, followed by freezing and cryosectioning or (c) freezing and cryosectioning. Staining was performed with one of the following Alizarin red S solutions: ARS 1 (pH 9), ARS 2 (pH 6.4) or ARS 3 (pH 4.8). Intensity of ARS staining is classified as: ○ – no staining; * – light staining; ** – moderate staining; *** – intense staining. 'Diffusion' marks diffusion artifacts. Where different staining intensities were obtained for mancae and adults, both results are presented separately: mancae / adults. Gray labeled fields mark the procedures that resulted in clearly differential staining of exoskeletal cuticle in comparison to other tissues, indicating their suitability for calcified exoskeleton localization.

Tabela 2: Povzetek rezultatov histokemijske reakcije z barvilom alizarin rdeče S pri marzupijskih mankah in odraslih rakih enakonožcih *P. scaber*: Vzorce smo fiksirali na pet različnih načinov: (a1) s fiksativom Carnoy, (a2) s 3.7 % nevtralno raztopino formaldehida, (a3) s 70 % etanolom, (b) s 3,7 % nevtralno raztopino formaldehida in zamrzovanjem ali (c) samo z zamrzovanjem. Za barvanje smo uporabili eno od naslednjih raztopin barvila alizarin rdeče S: ARS 1 (pH 9), ARS 2 (pH 6.4) ali ARS 3 (pH 4.8). Intenziteta ARS barvanja je označena z naslednjimi kategorijami: ○ – brez obarvanja; * – rahlo obarvanje; ** – zmerno obarvanje; *** – intenzivno obarvanje. 'Diffusion' označuje difuzijski artefakt. Kjer je bila intenziteta obarvanja različna pri mankah in odraslih, sta navedena oba rezultata ločeno na način: manke / odrasli. Sivo obarvana polja označujejo postopke, pri katerih se je eksoskeletna kutikula izrazito diferencialno obarvala v primerjavi z drugimi tkivi, kar kaže na to, da so primerni za lokalizacijo kalcificiranega eksoskeleta.

procedure	**(a) chemical fixation and paraffin sectioning**					
	(a1) Carnoy fixative		**(a2) 3.7% formaldehyde**		**(a3) 70% ethanol**	
staining solution	exoskeletal cuticle	other tissues	exoskeletal cuticle	other tissues	exoskeletal cuticle	other tissues
ARS 1 (pH 9)	*	*	***	○	***	**
ARS 2 (pH 6.4)	***	**	***	○	***	***
ARS 3 (pH 4.8)	***	**	***	○	***	***

procedure	**(b) 3.7% formaldehyde, freezing and cryosectioning**		**(c) freezing and cryosectioning**	
staining solution	exoskeletal cuticle	other tissues	exoskeletal cuticle	other tissues
ARS 1 (pH 9)	***	*	***	**/*
ARS 2 (pH 6.4)	*** diffusion	* /**	**/*** diffusion	*/**
ARS 3 (pH 4.8)	*** diffusion	*/**	**/*** diffusion	*/***

EDTA) a very faint red staining was noticeable (Supplementary fig. 4).

In cryosections of neutral buffered formaldehyde fixed and frozen samples (procedure b) a clearly differential staining of exoskeletal cuticle in adults and mancae was obtained with ARS 1 solution (pH 9) (Table 2, Supplementary fig. 5). Staining in ARS 2 (pH 6.4) and ARS 3 (pH 4.8) resulted in intensely red exoskeleton in adults and mancae, but in the sections of adult specimens considerable staining of other tissues was also evident. In addition, a diffuse red staining in the close vicinity of exoskeletal cuticle was observed in all samples subjected to ARS 2 or ARS 3 solutions. Diffusion of stain occurs as a consequence of calcium salts solubility in staining solutions and is termed diffusion artifact. Negative controls (pretreated in EDTA) showed no red staining in ARS 1 and a very faint colouring in ARS 2 and ARS 3 (Supplementary fig. 5).

In the specimens which were not chemically fixed and were frozen only (procedure c), staining was not clearly differential, except for the basic ARS staining (ARS 1) of adult cuticle, where exoskeleton was intensely red and only a light background was visible (Table 2, Supplementary fig. 6). In all other stainings applied to frozen only sections various difficulties regarding histochemical reaction were encountered: (i) staining of other tissues, (ii) diffusion artifacts and (iii) nonuniform staining of different slides or sections that was evident especially in mancae. Negative controls displayed no staining, only in sections of adults pretreated with EDTA and exposed to acid staining solution a faint colouring was visible (Supplementary fig. 6).

Discussion

Histological demonstration of calcified tissues is commonly performed by alizarin red S (ARS) and von Kossa's methods (Bancroft and Gamble 2008). Although a great variety of other more advanced and sophisticated methods for calcium demonstration is available, histological methods are beneficial for examination of numerous samples to gain preliminary coarse information about the calcified tissue localization. Von Kossa's method is not specific for the calcium cations, but depends on the presence of the salt anion (carbonate, phosphate, oxalate), while alizarin red S (sodium alizarin sulphonate) reacts with calcium (McGee-Russell 1958, Bancroft and Gamble 2008). It reacts also with other metallic cations like copper, magnesium, barium, zinc, iron, aluminium, etc, but generally they are not present in biological structures in sufficient quantities for histochemical demonstration (Puchtler et al. 1969, Lievremont et al. 1982). A great variety of alizarin red S method modifications is reported in the literature. Modifications involve mainly differences in tissue fixation and in pH of staining solution. Fixation methods and pH of staining solution have an effect on calcium preservation in tissue, staining sensibility and specificity. Acidity of ARS solution leads to releasing of more calcium ions from tissues (Puchtler et al. 1969, Kiernan 2008). Consequently calcium localization appears more dispersive and sensitivity for calcified tissue demonstration appears higher. Basic ARS solution has lower sensitivity but enables more precise localization of calcified tissue. ARS usually slightly stains the background tissue as well as it is an anionic dye (Puchtler et al. 1969, Kiernan 2008). Previous studies that include method of ARS staining are particularly based on vertebrates calcified tissues, while systematic evaluations of this method for other calcified biological systems rarely occur. Here we show a comparison of fifteen different modifications of ARS method applied to mancae and adult isopod crustaceans, to establish a quick, simple and inexpensive procedure appropriate to screen a large number of samples to estimate the presence of cuticle calcification.

The best differential staining of the exoskeletal cuticle in marsupial mancae and in adults as positive control was achieved with 3.7% formaldehyde fixation (2 days) and paraffin sections staining with ARS 1 (pH 9), ARS 2 (pH 6.4) or ARS 3 (pH 4.8) solution. The exoskeletal cuticle was specifically stained in all samples and the background remained unstained. Samples kept in neutral formaldehyde solution for a month, did not give positive staining of mancae cuticle (data not shown). Although formaldehyde is a widely recommended fixative, some authors described it as unsuitable fixative for studies of calcium deposits as it could remove some calcium during prolonged fixation (Puchtler et al. 1969, Presnell and Schreibmann 1997).

Alcoholic fixatives are widely recommended for calcified tissue localization as it is said they preserve calcium in tissue (Puchtler et al. 1969, Presnell and Schreibmann 1997, Bancroft and Gamble 2008, Kiernan 2008), though less adequate preservation of tissue structure could be caused by dehydrating effect. Since ARS staining of 70% ethanol fixed specimens in our study was not clearly specific, with intense or moderate staining of the background, we consider that 70% ethanol is not a suitable fixative for differential calcified cuticle localization. Staining of Carnoy fixed specimens was also not clearly differential, as in additon to exoskeletal cuticle all other tissues were stained too. We included this fixation in our study as it was recommended for vertebrate calcified tissues by Puchtler et al. (1969) and by Presnell and Schreibmann (1997). Our results also showed that all Carnoy fixed tissues were less intensely

stained in basic ARS solution in comparison to other two ARS solutions. We conclude that Carnoy fixative is not suitable for calcified cuticle identification.

Next, we performed cryosectioning to minimize exposure of samples to aqueous solutions, that could cause loss of amorphous forms of calcium from tissue as it is known that amorphous calcium carbonate has high solubility in water (Brečević and Nielsen 1989, Gal et al. 1996, Meiron et al. 2011). These methods keep most of the tissue components intact and are considered a better choice to study tissue composition, in spite of the fact that tissue structures appear poorly resolved. These methods are also less time consuming than conventional histological methods. Cryosections of samples fixed in neutral buffered formaldehyde and stained by basic ARS solution (ARS 1, pH 9) resulted in evidently differential staining of exoskeletal cuticle in marsupial mancae and in adults (a positive control). In all other procedures applied to specimens frozen after chemical fixation and to frozen only specimens, the histochemical reaction was either not differential or other imperfections were encountered, like diffusion artifacts and nonuniform staining. Diffusion artifacts, observed after staining in neutral and acid ARS solutions, were described also by Puchtler et al. (1969) in human tissues. Diffusion artifacts presumably appear due to the higher solubility of calcium salts in acid solutions in comparison to basic solutions. Nonuniform staining of sequential slides or sequential cryosections on the same slide, that we observed in the specimens of frozen only mancae, were possibly due to minimal variations in washing after staining.

Our results showed that cuticle of marsupial mancae was intensely and differentially stained by four different alizarin red S histochemical procedures, which also resulted in the differential staining of the exoskeletal cuticle in adults. These results suggest that prominent calcification of exoskeletal cuticle is present during postembryonic development of *P. scaber* mancae in the marsupium. Calcification provides hardness of exoskeleton that enables its protective role and mobility of the animal. Our findings show an importance of cuticle calcification for exoskeleton rigidity in mancae before they leave the marsupium. These findings support previous suggestions made by Surbida and Wright (2001) and by Ouyang and Wright (2005), that do not give direct evidence of cuticle calcification since the aims of these studies were focused to other issues, like investigations of osmotic tolerance and total calcium concentration in developmental stages. Surbida and Wright (2001) presume that wide osmotic tolerance of *Armadillidium vulgare* marsupial mancae is a consequence of calcification of their cuticle. Ouyang and Wright (2005) suggest that cuticle calcification starts in the stage of marsupial manca, as they observed the increase of total calcium concentration in isopod *Armadillidium vulgare* late-stage marsupial manca. In order to address the issue of calcium forms in marsupial mancae additional analytical methods for demonstration of mineral forms should be performed.

Conclusions

Exoskeletal cuticle of marsupial mancae and adults of *P. scaber* was differentially stained by the following varieties of the histochemical alizarin red S method:

(a) in paraffin sections of formaldehyde fixed samples, stained with alizarin red S solutions ARS 1 (pH 9), ARS 2 (pH 6.4) or ARS 3 (pH 4.8) and
(b) in cryosections of samples fixed in formaldehyde, stained with basic ARS solution (ARS 1, pH 9).

This study suggests that prominent calcification of exoskeletal cuticle occurs already in marsupial mancae of isopod crustacean *P. scaber*. Exoskeleton hardening is likely important also for body movements, that we observed in mancae before they were released from marsupium.

Alizarin red S procedures that resulted in distinct differential staining of exoskeletal cuticle in this study are expected to be applicable for localization of calcified chitinous matrices in other species.

Acknowledgements

This work was supported by the Slovenian Research Agency (ARRS), grant No. 1000-11-310087. We would like to thank Jožica Murko Bulić

for her laboratory assistance and the reviewers for their constructive suggestions.

Povzetek

Eksoskeletna kutikula rakov je apikalni zunajcelični matriks epidermisa, osnovan na hitinskem organskem ogrodju in utrjen s sklerotizacijo in kalcifikacijo. Tvorba nove kutikule poteka v zgodnjem razvoju in ob vsaki levitvi pri odraslih osebkih. Pomemben proces v formiranju nove kutikule je tudi kalcifikacija, nalaganje kalcijevih mineralov v organski matriks. Embriji kopenskega raka enakonožca vrste *Porcellio scaber* se razvijajo v vodnem okolju valilnika (marzupija), ki je vrečasta struktura na trebušni strani samice. Embriji se izležejo v ličinke manke, ki nadaljujejo razvoj v valilniku še približno teden dni. Predhodno smo ugotovili, da kutikula marzupijskih mank kaže osnovne ultrastrukturne značilnosti kutikule odraslih živali, kot so organizacija v glavne sloje – epikutikulo, eksokutikulo in endokutikulo ter razporeditev hitinsko – proteinskih vlaken v značilen vzorec (Mrak in sod. 2012). V tej študiji smo ugotavljali, ali je eksoskeletna kutikula pri marzupijskih mankah že kalcificirana. V ta namen smo uporabili histokemijsko tehniko za lokalizacijo kalcificiranega tkiva z barvilom alizarin rdeče S (ARS), ki omogoča preprost in hiter pregled večjega števila vzorcev in je osnova za nadaljne bolj zahtevne in natančne tehnike. Glede na to, da je v literaturi predlaganih več modifikacij te metode, ki se primarno uporablja v histologiji vretenčarjev, smo izvedli pet različnih načinov fiksacije tkiva: (a1) v fiksativu Carnoy, (a2) v 3,7 % nevtralni raztopini formaldehida ali (a3) v 70 % etanolu, (b) fiksacija v 3,7 % nevtralni raztopini formaldehida in zamrzovanje ali (c) samo zamrzovanje. Barvali smo s tremi različnimi raztopinami barvila: ARS 1 (pH 9), ARS 2 (pH 6,4) ali ARS 3 (pH 4,8). Za pozitivno kontrolo smo uporabili barvanje eksoskeletne kutikule odraslih živali, za negativo kontrolo pa predhodno inkubacijo rezin odraslih živali in mank v dekalcifikacijski raztopini EDTA. Eksoskeletna kutikula marzupijskih mank in odraslih živali se je izrazito diferencialno obarvala pri vzorcih fiksiranih v nevtralni raztopini formaldehida, vklopljenih v parafin in barvanih z eno izmed raztopin barvila alizarin rdeče S: ARS 1 (pH 9), ARS 2 (pH 6,4) ali ARS 3 (pH 4,8). Da bi se čimbolj izognili vodnim raztopinam, v katerih so amorfne oblike kalcijevih soli dobro topne, smo v študijo vključili fiksacijo vzorcev z zamrzovanjem in barvanje kriostatskih rezin. Pri tej tehniki se je eksoskeletna kutikula diferencialno obarvala v primeru zamrznjenih rezin vzorcev, predhodno fiksiranih v formaldehidu, ki smo jih barvali z bazično raztopino ARS 1 (pH 9). Postopki histokemijske lokalizacije z barvanjem ARS, ki so se izkazali kot primerni, bodo predvidoma uporabni tudi pri študijah kalcifikacije drugih hitinskih matriksov.

Eksoskeletna kutikula marzupijskih mank *P. scaber* se je izrazito diferencialno obarvala s štirimi različnimi postopki metode ARS, pri katerih smo enako diferencialno obarvanje dobili tudi v primerih eksoskeletne kutikule pri odraslih (pozitivne kontrole). Ti rezultati kažejo na znatno kalcifikacijo eksoskeletne kutikule že v razvojnem obdobju pred sprostitvijo v zunanje okolje. Eksoskelet se torej oblikuje in kalcificira že pri ličinkah mankah v marzupiju, kar je najverjetneje pomembno tudi za gibanje mank, ki smo ga opazili pred sprostitvijo iz valilnika. Za ugotavljanje oblik kalcijevih soli v eksoskeletu marzupijskih mank bi bilo v nadaljevanju dela potrebno uporabiti analitske metode za identifikacijo mineralnih oblik, kot sta npr. infrardeča in Raman spektroskopija.

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Supplementary material

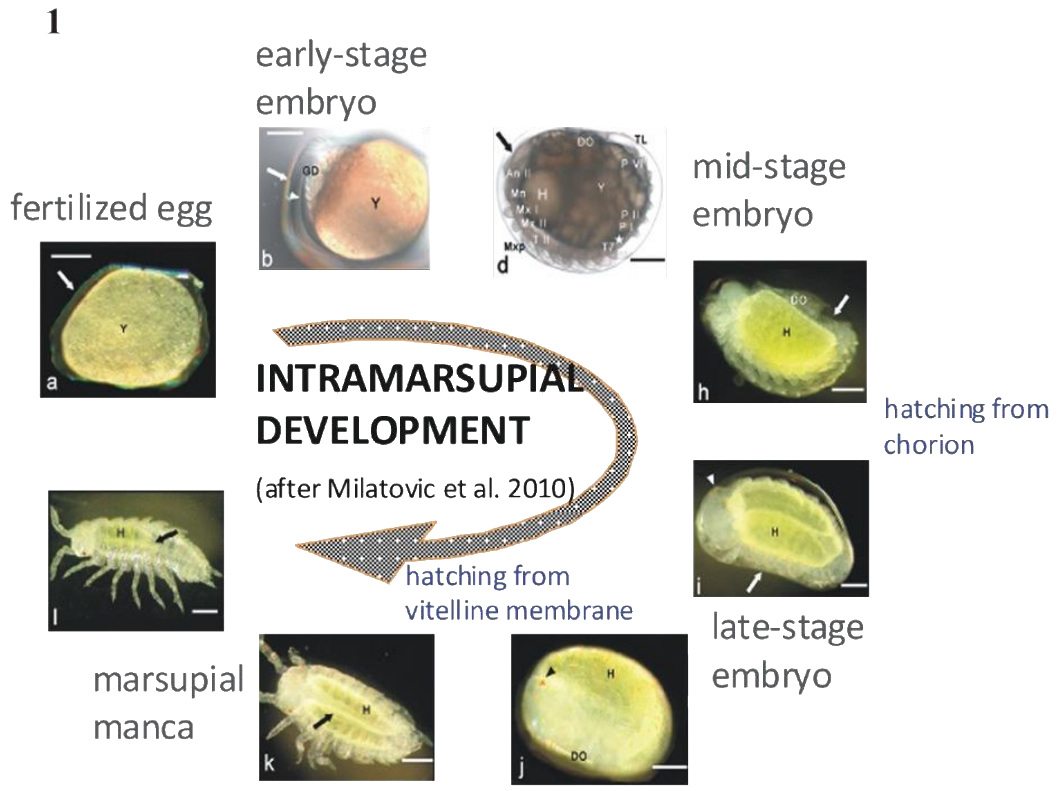

Figure 1: Principal stages of *P. scaber* intramarsupial development, from fertilized egg, early-, mid- and late-stage embryos to marsupial manca (adapted from staging system in Milatovič et al. (2010)).

Slika 1: Glavni stadiji razvoja raka *P. scaber* v marzupiju, od oplojenega jajčeca, zgodnjega, srednjega in poznega embrija do marzupijske manke (povzeto po razvojnem sistemu v Milatovič in sod. (2010)).

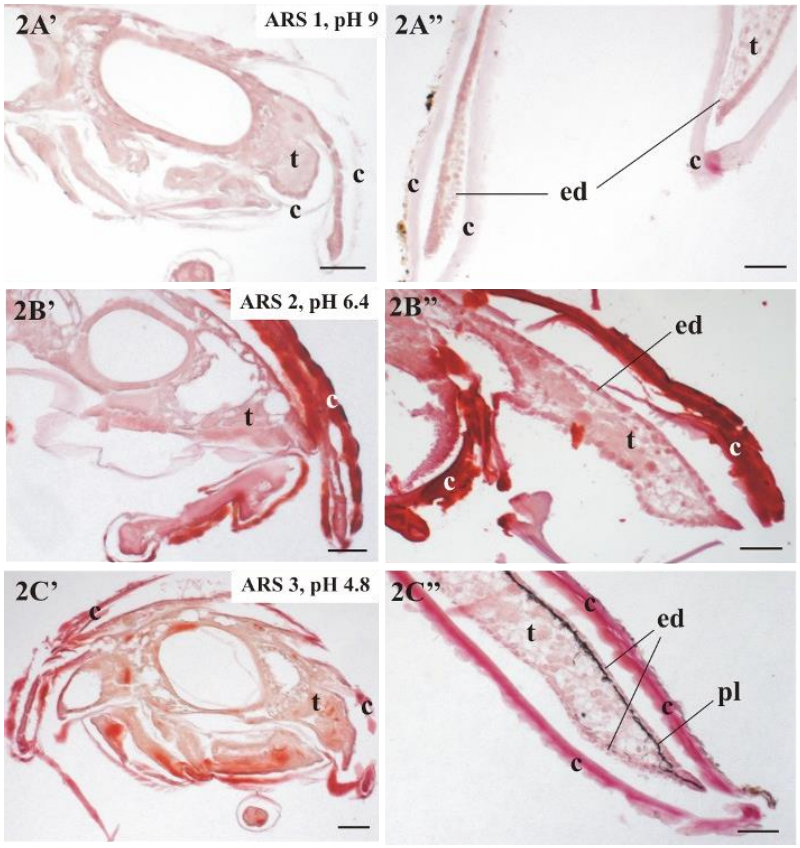

Figure 2: Alizarin red S staining of *P. scaber* specimens, fixed in Carnoy fixative and paraffin embedded (procedure a1). A', A" - Staining solution ARS 1 (pH 9). B', B" - Staining solution ARS 2 (pH 6.4). C', C" - Staining solution ARS 3 (pH 4.8). A', B', C' - Marsupial mancae, cross sections. A", B", C" – Positive controls - adults, cross sections. Staining of exoskeletal cuticle was not differential, neither in mancae nor in adults. c – cuticle; ed – epidermis; pl – pigment layer; t – tissue. Bars: 50 µm.

Slika 2: Histokemijska lokalizacija z barvilom alizarin rdeče S - barvanje vzorcev rakov enakonožcev *P. scaber*, fiksiranih v fiksativu Carnoy in vklopljenih v parafin (postopek a1). A', A" – Barvanje v raztopini ARS 1 (pH 9). B', B" - Barvanje v raztopini ARS 2 (pH 6.4). C', C" - Barvanje v raztopini ARS 3 (pH 4.8). A', B', C' – Marzupijske manke, prečni prerezi. A", B", C" – Pozitivne kontrole - odrasle živali, prečni prerezi. Tako pri mankah kot pri odraslih eksoskeletna kutikula ni bila diferencialno obarvana. c – kutikula; ed – epidermis; pl – pigmentni sloj; t – tkivo. Merila: 50 µm.

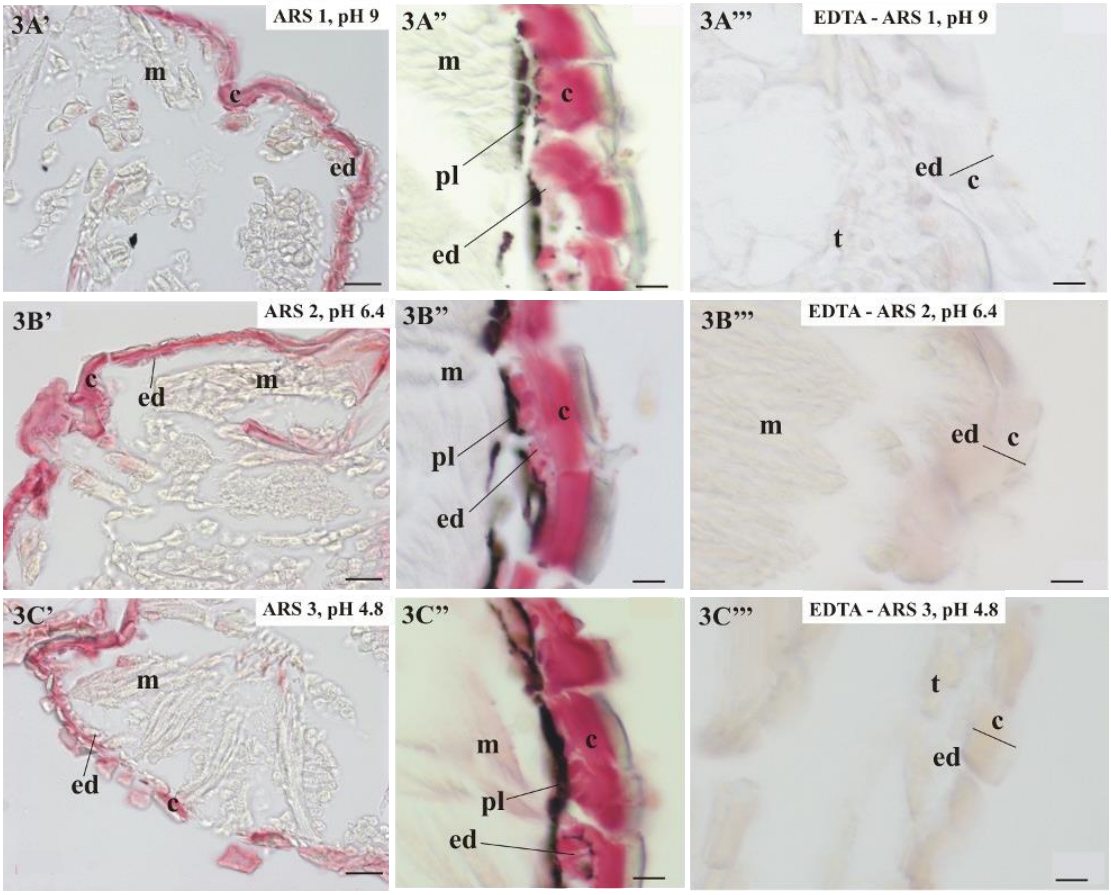

Figure 3: Alizarin red S staining of *P. scaber* specimens, fixed in neutral buffered formaldehyde and paraffin embedded (procedure a2). A', A", A'" - Staining solution ARS 1 (pH 9). B', B", B'" - Staining solution ARS 2 (pH 6.4). C', C", C'" - Staining solution ARS 3 (pH 4.8). A', B', C' – Marsupial mancae, cross sections. A", B", C" – Positive controls - adults, dorsal regions of animals cross sections. Exoskeletal cuticle was clearly differentially stained in mancae and adults with all three staining variations. C', C" - Negligible colouring of other tissues was visible in ARS 3 staining. A'", B'", C'" - Negative controls in adults (EDTA) resulted in negligible or no staining. c – cuticle; ed – epidermis; m – muscles; pl – pigment layer; t – tissue. Bars: A', B', C' – 20 µm; A", A'", B", B'", C", C'" – 10 µm.

Slika 3: Histokemijska lokalizacija z barvilom alizarin rdeče S - barvanje vzorcev rakov enakonožcev *P. scaber*, fiksiranih v nevtralni raztopini formaldehida in vklopljenih v parafin (postopek a2). A', A", A'" – Barvanje v raztopini ARS 1 (pH 9). B', B", B'" – Barvanje v raztopini ARS 2 (pH 6.4). C', C", C'" – Barvanje v raztopini ARS 3 (pH 4.8). A', B', C' – Marzupijske manke, prečni prerezi. A", B", C" – Pozitivne kontrole - odrasle živali, dorzalni predeli živali v prečnem prerezu. Eksoskeletna kutikula se je diferencialno obarvala pri mankah in odraslih pri vseh treh različicah barvanja. C', C" – Pri barvanju z raztopino ARS 3 so se zanemarljivo obarvala tudi druga tkiva. A'", B'", C'" – Negativne kontrole odraslih (EDTA) se niso obarvale ali pa je bilo obarvanje zanemarljivo. c – kutikula; ed – epidermis; m – mišice; pl – pigmentni sloj; t – tkivo. Merila: A', B', C' – 20 µm; A", A'", B", B'", C", C'" – 10 µm.

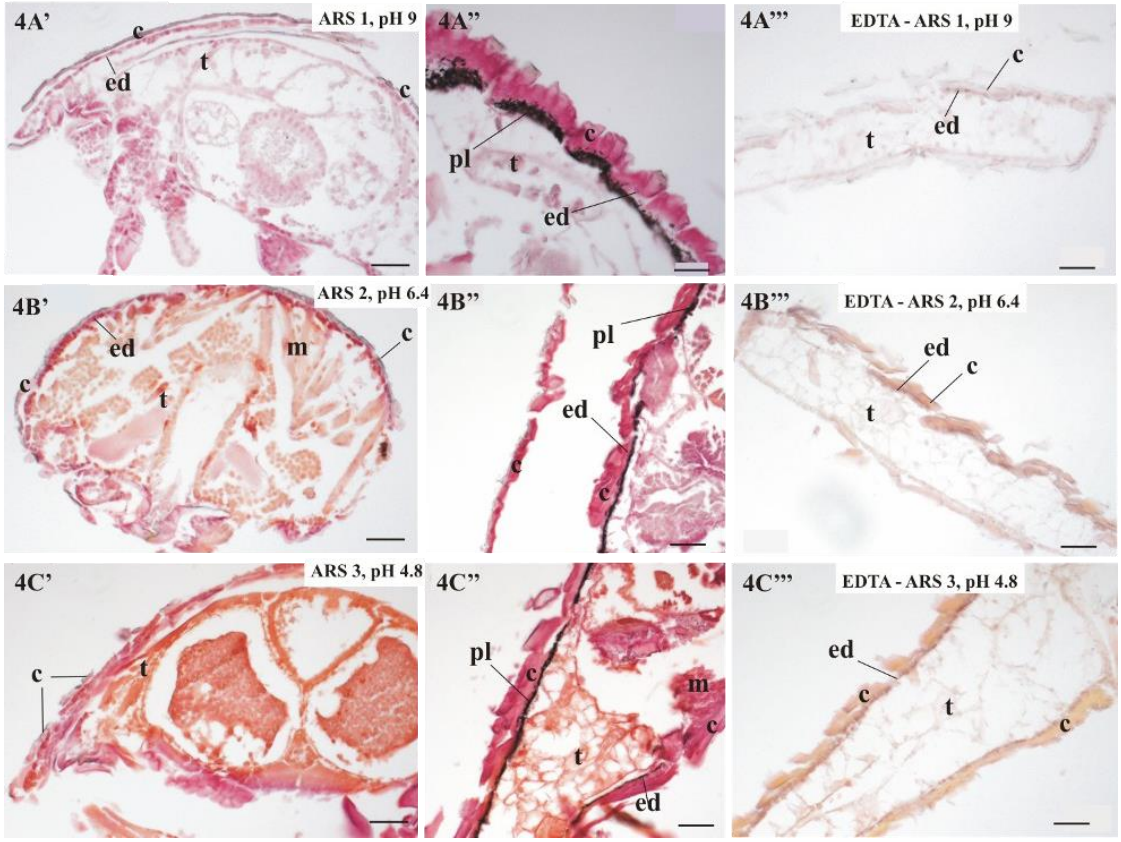

Figure 4: Alizarin red S staining of *P. scaber* specimens, fixed in 70% ethanol and paraffin embedded (a3). A', A", A"' - Staining solution ARS 1 (pH 9). B', B", B"' - Staining solution ARS 2 (pH 6.4). C', C", C"' Staining solution ARS 3 (pH 4.8). A', B', C' - Marsupial mancae, cross sections. A" B", C" – Positive controls - adults, cross sections. In ARS 1 staining of adults, exoskeletal cuticle was more intensely stained than other tissues (A"). In all other samples exoskeletal cuticle and other tissues were similarly stained. A"', B"', C"' - Staining of negative controls in adults (EDTA) resulted in a very faint red colouring. c – cuticle; ed – epidermis; m – muscles; pl – pigment layer; t – tissue. Bars: 50 µm.

Slika 4: Histokemijska lokalizacija z barvilom alizarin rdeče S - barvanje vzorcev rakov enakonožcev *P. scaber*, fiksiranih v 70% etanolu in vklopljenih v parafin (postopek a3). A', A", A"' – Barvanje v raztopini ARS 1 (pH 9). B', B", B"' – Barvanje v raztopini ARS 2 (pH 6.4). C', C", C"' – Barvanje v raztopini ARS 3 (pH 4.8). A', B', C' - Marzupijske manke, prečni prerezi. A" B", C" – Pozitivne kontrole - odrasle živali, prečni prerezi. Pri barvanju odraslih živali z raztopino ARS 1 se je eksoskeletna kutikula obarvala bolj intenzivno kot druga tkiva (A"). Pri ostali vzorcih so bila eksoskeletna kutikula in ostala tkiva podobno obarvana. A"', B"', C"' – Negativne kontrole odraslih (EDTA) so se obarvale zelo šibko. c – kutikula; ed – epidermis; m – mišice; pl – pigmentni sloj; t – tkivo. Merila: 50 µm.

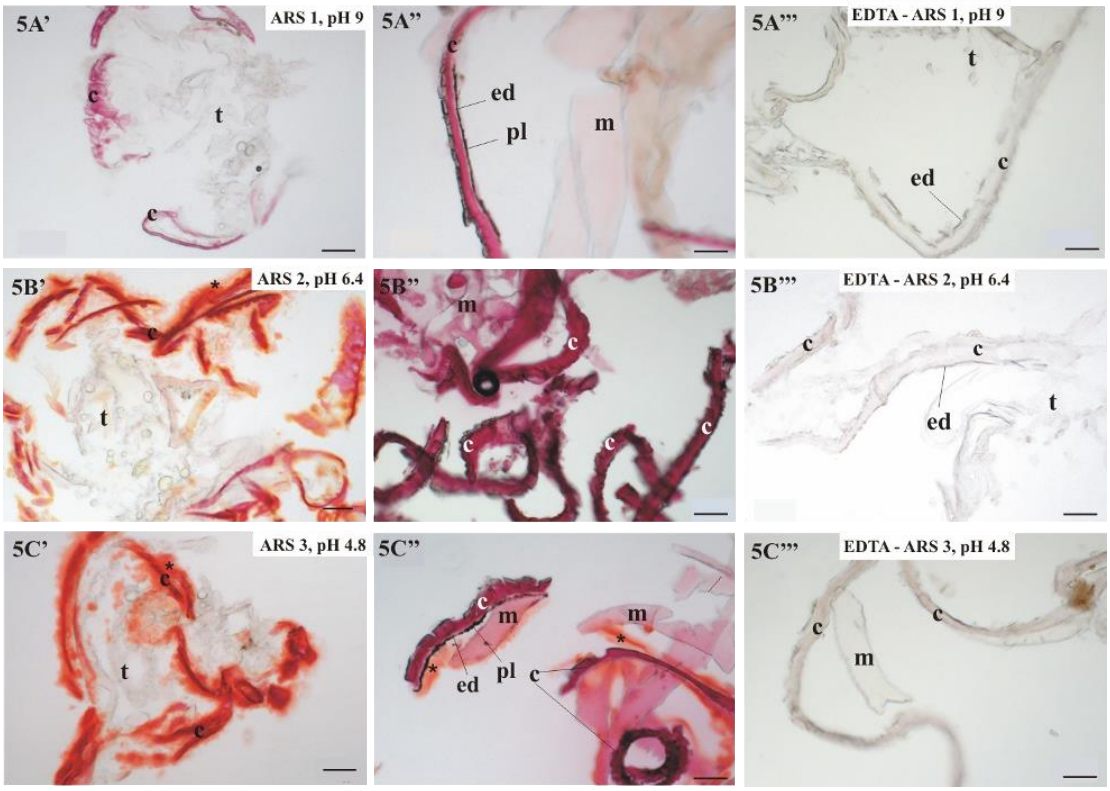

Figure 5: Alizarin red S staining of *P. scaber* specimens, fixed in neutral buffered formaldehyde and cryosectioned (procedure b). A', A", A'" - Staining solution ARS 1 (pH 9). B', B", B'" - Staining solution ARS 2 (pH 6.4). C', C", C'" - Staining solution ARS 3 (pH 4.8). Structure of the tissues was not clearly distinctive, but cuticle and muscles were recognizable. A', B', C' – Marsupial mancae, cross sections. A", B", C" – Positive controls – adults, cross sections. In ARS 1 solution exoskeletal cuticle of mancae and adults was clearly differentially stained (A', A"). In ARS 2 (B', B") and ARS 3 (C', C") solutions exoskeletal cuticle of mancae and adults was intensely red stained and diffusion artifacts (*) were present. In adults other tissues were stained also (B", C"). A'", B'", C'" - Negative controls in adults (EDTA) were not red stained, only a faint colouring was noticeable with ARS 2 and ARS 3 staining. c – cuticle; ed – epidermis; m – muscles; pl – pigment layer; t – tissue. Bars: 50 µm.

Slika 5: Histokemijska lokalizacija z barvilom alizarin rdeče S - barvanje zamrznjenih vzorcev rakov enakonožcev *P. scaber*, ki so bili predhodno fiksirani v nevtralni raztopini formaldehida (postopek b). A', A" A'"– Barvanje v raztopini ARS 1 (pH 9). B', B", B'" – Barvanje v raztopini ARS 2 (pH 6.4). C', C", C'" – Barvanje v raztopini ARS 3 (pH 4.8). Struktura tkiv ni bila povsem razločna, prepoznavne so bile kutikula in mišice. A', B, C' – Marzupijske manke, prečni prerezi. A", B", C" – Pozitivne kontrole - odrasle živali, prečni prerezi. V ARS 1 raztopini se je eksoskeletna kutikula mank in odraslih diferencialno obarvala (A', A"). Pri barvanjih z ARS 2 (B', B") in ARS 3 (C', C") raztopinama se je kutikula mank in odraslih intenzivno rdeče obarvala. Opazni so bili difuzijski artefakti (*). Pri odraslih so se obarvala tudi druga tkiva (B", C"). A'", B'", C'" - Negativne kontrole odraslih (EDTA) niso bile rdeče obarvane, opazno je bilo le šibko obarvanje v raztopinah ARS 2 in ARS 3. c – kutikula; ed – epidermis; m – mišice; pl – pigmentni sloj; t – tkivo. Merila: 50 µm.

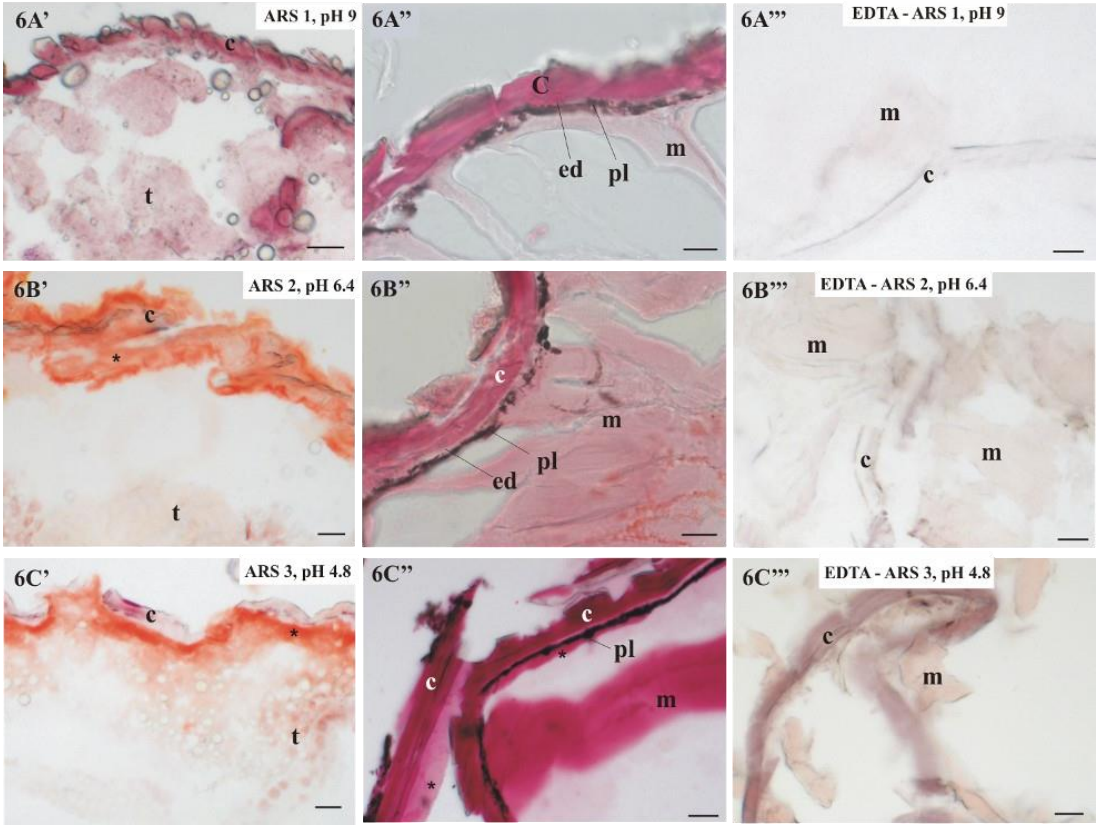

Figure 6: Alizarin red S staining of *P. scaber* specimens, frozen and cryosectioned (procedure c). A', A", A"' - Staining solution ARS 1 (pH 9). B', B", B"' - Staining solution ARS 2 (pH 6.4). C', C", C"' - Staining solution ARS 3 (pH 4.8). Histological structure was not well preserved, although cuticle and muscles were recognizable. A', B', C' – Marsupial mancae, longitudinal sections. A", B", C" – Positive controls - adults, cross sections. In ARS 1 staining of adults the exoskeletal cuticle was differentially stained (A"). In ARS 2 (B', B") and ARS 3 stainings (C', C") diffusion artifacts (*) were present in all samples and considerable background was evident in adults. In mancae a nonuniformity of staining was also observed. A"', B"', C"' - Negative controls of adults (EDTA) resulted in no staining, only a faint colouring was visible in ARS 3 staining. c – cuticle; ed – epidermis; m – muscles; pl – pigment layer; t – tissue. Bars: 20 µm.

Slika 6: Histokemijska lokalizacija z barvilom alizarin rdeče S - barvanje zamrznjenih vzorcev rakov enakonožcev *P. scaber* (postopek c). A', A", A"' – Barvanje v raztopini ARS 1 (pH 9). B', B", B"' – Barvanje v raztopini ARS 2 (pH 6.4). C', C", C"' – Barvanje v raztopini ARS 3 (pH 4.8). Struktura tkiv je bila histološko slabo ohranjena, prepoznavne so bile kutikula in mišice. A', B', C' – Marzupijske manke, vzdolžni prerezi. A", B", C" – Pozitivne kontrole - odrasle živali, prečni prerezi. Pri ARS 1 barvanju odraslih živali se je eksoskeletna kutikula diferencialno obarvala (A"). Pri barvanju v raztopinah ARS 2 (B', B") in ARS 3 (C', C") so se pri vseh vzorcih pojavili difuzijski artefakti (*). Pri odraslih živalih je bilo ozadje precej obarvano. Pri barvanju mank je bila intenziteta barvanja neenakomerna. A"', B"', C"' – Negativne kontrole odraslih (EDTA) se niso obarvale, opazno pa je bilo šibko obarvanje pri ARS 3 raztopini. c – kutikula; ed – epidermis; m – mišice; pl – pigmentni sloj; t – tkivo. Merila: 20 µm.

2.1.3 Diferenciacija eksoskeletne kutikule med razvojem v valilniku pri raku enakonožcu *Porcellio scaber* (Crustacea: Isopoda)

Exoskeletal cuticle differentiation during intramarsupial development of *Porcellio scaber* (Crustacea: Isopoda)

Polona Mrak, Nada Žnidaršič, Kristina Žagar, Miran Čeh in Jasna Štrus

Arthropod Structure and Development, 2014, 423-439

V članku smo opisali in pojasnili funkcionalno ultrastrukturo prekutikularnih matriksov in eksoskeletne kutikule med razvojem embrijev in ličink v valilniku pri raku enakonožcu *Porcellio scaber*. Poleg tega smo v apikalnih epidermalnih matriksih lokalizirali makromolekule, ki vsebujejo *N*- acetilglukozamin, kar vključuje tudi hitin kot osnovno organsko komponento kutikule. Lokalizacijo smo izvedli na nivoju elektronske mikroskopije, z uporabo lektina WGA ('wheat germ agglutinin'), konjugiranega z zlatimi zrni. Z metodo SEM-EDXS smo analizirali elementno sestavo epidermalnih matriksov v različnih razvojnih stadijih. V članku smo opisali tudi modifikacije apikalne plazmaleme, ki so povezane s sintezo in diferenciacijo apikalnih matriksov.

Prve znake odlaganja apikalnega matriksa smo opazili pri srednjem embriju v stadiju 10, kjer je nad izboklinami apikalne plazmaleme viden tanek sloj materiala. Epidermis srednjega embrija v nadaljnjem razvoju tvori najmanj dva prekutikularna matriksa. Prekutikularni matriks sestavljata elektronsko gosta lamina in rahel material pod njo. Struktura in rezultati WGA označevanja kažejo, da ima prekutikularni matriks drugačno organsko ogrodje kot kutikula odraslih živali. Prekutikularna matriksa se odluščita s površine embrija med prehodom iz srednjega v pozni embrij, ko se embrij izleže iz horiona, in v obdobju izleganja embrija iz vitelinske membrane. Najbolj zgodnji razvojni stadij, pri katerem je že oblikovana kutikula iz epikutikule in prokutikule, je pozni embrij pred izleganjem iz vitelinske membrane. Epikutikula ima petslojno strukturo, podobno epikutikuli odraslih živali, in tvori kutikularne luske. Prokutikula izkazuje izrazito vezavo lektina WGA, kar kaže na podobnost z organskim matriksom diferencirane kutikule. Do konca embrionalnega razvoja se kutikula odebeli in diferencira v epi-, ekso- in endokutikulo s podsloji. Prva levitev poteče približno v obdobju izleganja embrija iz vitelinske membrane. Iz vitelinske membrane izležena marzupijska ličinka manka tvori novo kutikulo, v kateri smo ugotovili nalaganje kalcija. Razmerje spektralnih vrhov kalcija in fosforja je manjše od 1, v primerjavi z značilnim visokim razmerjem vrhov kalcija in fosforja pri kutikuli odraslih, kjer je kalcijev vrh nekajkrat višji od fosforjevega. V nadaljnjem razvoju ličinke manke v valilniku se kutikularni sloji strukturno diferencirajo, kutikula se odebeli, kalcifikacija kutikule pa je bolj izrazita. Pri napredni zgodnji marzupijski manki je kutikula tudi po elementni

sestavi in vezavi lektina WGA podobna kutikuli odrasle živali. Pri poznih marzupijskih mankah smo opazili odmik kutikule od epidermisa, razgradnjo notranjih endokutikularnih slojev in tvorbo nove kutikule, kar kaže na obnavljanje eksoskeleta v valilniku in na levitev manke kmalu po sprostitvi iz valilnika. Rezultati kažejo, da menjava teh prehodnih površinskih matriksov časovno sovpada z obdobji intenzivne rasti in morfološkimi spremembami ter verjetnimi spremembami v osmoregulacijski kapaciteti embrijev in mank. V epidermalnih celicah, ki jih pokriva prekutikularni matriks ali nastajajoča kutikula, smo opazili preoblikovanje apikalne plazmaleme v nizke izbokline z elektronsko gostimi konicami. Pri sintezi nove kutikule marzupijskih mank so opazni tudi široki citoplazemski izrastki, ki segajo globoko v kutikulo. Strukturna organiziranost in sestava kutikule pri marzupijskih ličinkah, ki je že podobna kutikuli odraslih živali, nakazuje pomembno vlogo eksoskeleta pri zaščiti in opori telesa ličinke med gibanjem v valilniku in med sprostitvijo v zunanje okolje.

Arthropod Structure & Development 43 (2014) 423–439

Contents lists available at ScienceDirect

Arthropod Structure & Development

journal homepage: www.elsevier.com/locate/asd

Exoskeletal cuticle differentiation during intramarsupial development of *Porcellio scaber* (Crustacea: Isopoda)

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ARTICLE INFO

Article history:
Received 31 March 2014
Accepted 9 July 2014
Available online 19 July 2014

Keywords:
Embryonic development
Larva manca
Ultrastructure
Chitin
Calcification
Precuticular matrix

ABSTRACT

Exoskeletal crustacean cuticle is a calcified apical extracellular matrix of epidermal cells, illustrating the chitin-based organic scaffold for biomineralization. Studies of cuticle formation during molting reveal significant dynamics and complexity of the assembly processes, while cuticle formation during embryogenesis is poorly investigated. This study reveals in the terrestrial isopod *Porcellio scaber*, the ultrastructural organization of the differentiating precuticular matrices and exoskeletal cuticles during embryonic and larval intramarsupial development. The composition of the epidermal matrices was obtained by WGA lectin labelling and EDXS analysis. At least two precuticular matrices, consisting of loosely arranged material with overlying electron dense lamina, are secreted by the epidermis in the mid-stage embryo. The prehatching embryo is the earliest developmental stage with a cuticular matrix consisting of an epicuticle and a procuticle, displaying WGA binding and forming cuticular scales. In newly hatched marsupial larva manca, a new cuticle is formed and calcium sequestration in the cuticle is evident. Progression of larval development leads to the cuticle thickening, structural differentiation of cuticular layers and prominent cuticle calcification. Morphological characteristics of exoskeleton renewal in marsupial manca are described. Elaborated cuticle in marsupial larvae indicates the importance of the exoskeleton in protection and support of the larval body in the marsupium and during the release of larvae in the external environment.

1. Introduction

The exoskeletal cuticle of arthropods is a chitinous extracellular matrix, formed apically by epidermal cells. Cuticle is a highly elaborated structure with different constituents, such as polysaccharide chitin, proteins and lipids organized in intricate layers (Neville, 1984; Compere et al., 2004; Dillaman et al., 2013). Epicuticle is the thin outermost layer, consisting of lipids and proteins. Procuticle is the inner protein–chitin matrix that is further divided into exocuticle and basal endocuticle, which differ in ultrastructure and contrast. This complex organization enables the cuticle to perform various functions including protection against dehydration, predators and pathogens, interaction with the environment, mechanical support of the body and, in connection with the muscular system, assistance in locomotion. Formation of the cuticle during embryogenesis has been investigated primarily in insects (Dorn, 1976; Ziese and Dorn, 2003; Konopova and Zrzavy, 2005; Moussian et al., 2006). Molecular and genetic issues of cuticle differentiation have also been addressed in studies of insects' embryos, mostly in the model species *Drosophila melanogaster* (Ostrowski et al., 2002; Payre, 2004; Moussian et al., 2005, 2007; Andrew and Baker, 2008; Moussian, 2010). The structure and composition of crustacean cuticle and its structural reorganization during molting in adults of different crustaceans, including isopods, are well known (Price and Holdich, 1980; Roer and Dillaman, 1984; Compere and Goffinet, 1987a,b; Compere, 1990; Štrus and Compere, 1996; Ziegler, 1997; Štrus and Blejec, 2001; Dillaman et al., 2005; Neues et al., 2011; Vittori et al., 2012). Several data on the structure of the crustacean cuticle during development have been derived from decapods, branchiopods and amphipods. Many of the early studies are focused on *in vivo* or histological observations of the larval molt cycle (Freeman and Costlow, 1980; Anger, 1983; Snyder and Chang, 1986). Embryonic cuticles and embryonic molt cycles were observed by light microscopy in intact and

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http://dx.doi.org/10.1016/j.asd.2014.07.002
1467-8039/

unfixed embryos, for example in the decapod *Homarus americanus* (Helluy and Beltz, 1991) and in the branchiopod *Eulimnadia antlei* (Belk, 1987). However, ultrastructural studies, probing the cuticle in embryos and larvae are scarce, although some early studies were made on the ultrastructure of embryonic surface matrices in the branchiopod *Artemia salina* (Morris and Afzelius, 1967), in the isopod *Hemioniscus balani* (Goudeau, 1976) and in the decapods *Carcinus maenas* (Goudeau and Lachaise, 1983) and *Palaemonetes pugio* (Glas et al., 1997). An ultrastructural study of the embryo cuticle was performed recently in the aquatic amphipod *Parhyale hawaiensis* (Havemann et al., 2008). These studies were all focused only on the matrix secretion before hatching.

In comparisons of insect and crustacean exoskeletal cuticles, some common features in overall cuticle architecture and chitin-based structural framework are apparent, but on the other hand cuticles differ considerably in composition. Insect cuticle is sclerotized, while crustacean cuticle is not only sclerotized but hardened by calcification. Crustacean cuticle, once calcified, includes a crystalline polymorph of calcium carbonate (calcite) and amorphous polymorphs – amorphous calcium carbonate (ACC) and amorphous calcium phosphate (ACP) (Roer and Dillaman, 1984; Becker et al., 2005; Dillaman et al., 2005; Hild et al., 2008, 2009; Neues et al., 2011; Seidl et al., 2011; Luquet, 2012). The mineral component in crustacean exo- and endocuticle is incorporated in the organic matrix, thus enhancing its strength and stiffness. The elemental composition of the adult crustacean cuticle has been examined by atomic absorption spectroscopy (AAS) and by energy dispersive X-ray spectroscopy (EDXS) in several studies. Carbon, oxygen and calcium were demonstrated to be the major elements of crustacean cuticle and phosphorus and magnesium are also principal elements (Compere et al., 1992; Becker et al., 2005; Romano et al., 2007; Hild et al., 2008, 2009; Seidl et al., 2011; Ruangchai et al., 2013). In the cuticle, most phosphorus occurs in ACP (Becker et al., 2005; Luquet, 2012) and a significant proportion of magnesium occurs within the calcite crystal lattice as magnesium calcite (Becker et al., 2005; Hild et al., 2008, 2009). Calcification of the forming cuticle during crustacean development has not been investigated.

The species *Porcellio scaber* is a terrestrial isopod crustacean (Oniscidea), which possesses highly derived adaptations to land habitats. One significant adaptation is embryonic development in marsupium, the fluid-filled brood pouch on the ventral side of the female body that provides a controlled aqueous environment and protection for developing embryos. After hatching of embryos from both egg envelopes, chorion and vitelline membrane, larvae termed marsupial mancae develop in the marsupium for one week and emerge to the external environment as mancae with six pairs of pereopods (Wolff, 2009; Milatovič et al., 2010). Postmarsupial mancae develop a seventh pair of pereopods with progressive molts (Tomescu and Craciun, 1987). Under laboratory conditions, intramarsupial development of *P. scaber*, from fertilized eggs, embryos and marsupial mancae lasts approximately 35 days (Milatovič et al., 2010) and has been described as proceeding in twenty morphologically different stages (Wolff, 2009; Milatovič et al., 2010), but cuticle differentiation was not followed in detail.

This paper reports the ultrastructural characteristics of the exoskeletal cuticle during intramarsupial development of the terrestrial isopod *P. scaber*. A description of embryonic pre-cuticular matrices, deposited before formation of typical exoskeletal crustacean cuticle is an important new contribution. The cuticle apolysis and degradation as well as the new cuticle formation in marsupial manca are described, indicating that the exoskeleton of the marsupial mancae is renewed. In addition, information on the composition of apical epidermal matrices in different stages of intramarsupial development is presented. As chitin is the main structural molecule of the cuticle in adults and the composition of the embryonic epidermal matrices is unknown, labelling with lectin wheat germ agglutinin was performed to localize *N*-acetyl-glucosamine oligomers, including chitin, at the level of electron microscopy. The elemental composition of the epidermal matrices was examined by energy dispersive X-ray spectroscopy (EDXS) in the scanning electron microscope (SEM) to determine if matrix calcification starts during intramarsupial development.

2. Material and methods

2.1. Specimens of *P. scaber*

Specimens of *P. scaber* Latreille, 1809 (Crustacea: Isopoda) were maintained in a laboratory environment, in soil and leaf litter, at 25 °C, high relative humidity and at 12-h light/12-h dark cycle. Embryos and mancae were isolated from the marsupia of gravid females. Different developmental stages of embryos were identified according to Milatovič et al. (2010) and the marsupial mancae were classified according to Mrak et al. (2012). The following stages of embryos were used in this study: mid-stage embryo (stages 10, 13, 14 and 15) and late-stage embryo (stages 16, 18 and 19). To refine the crucial stage after embryo hatching to manca, we additionally subdivided the stage of early marsupial manca into two substages: newly hatched early manca and advanced early manca. The term "newly hatched early manca" defines 1.4–1.5 mm long, often slightly bent manca with no locomotion and widely extended midgut yolk, while the term "advanced early manca" is used for unbent manca of about 1.6 mm body length, in which motion of the pereopods is already observed. The subjected stages of marsupial mancae, listed in sequential order, are: newly hatched early marsupial manca, advanced early marsupial manca, mid-stage marsupial manca and late marsupial manca. In addition, adult *P. scaber* cuticle was used as positive control for analysing chitin localization and elemental composition. Adult animals that showed no external signs of molting were anaesthetized with diethylether. Tergites were isolated and processed simultaneously with the embryos and mancae.

2.2. Transmission electron microscopy

The ultrastructure of surface matrices is presented in mid-stage embryos, late embryos and marsupial mancae (Table 1). The egg envelopes of embryos were carefully perforated with a thin needle

Table 1
Overview of developmental stages and methods applied in this study.

Developmental stage		Method[a] (number of animals analysed)		
Mid-stage embryos	Stage 10	TEM (2)		
	Stage 13	TEM (1)		
	Stage 14	TEM (2)		
	Stage 15	TEM (2)		
Late-stage embryos	Stage 16	TEM (7)	WGA labelling (2)	
	Stage 18	TEM (4)	WGA labelling (2)	
	Stage 19	TEM (2)		
Marsupial mancae	Newly hatched	TEM (3)	WGA labelling (1)	EDXS (4)
	Advanced early	TEM (3)	WGA labelling (3)	EDXS (6)
	Mid-stage	TEM (1)		
	Late-stage	TEM (5)	WGA labelling (2)	

[a] The abbreviations used for the methods: TEM – ultrastructure analysis with a transmission electron microscope; WGA labelling – ultrathin sections labelling with lectin wheat germ agglutinin–gold probes and imaging with a transmission electron microscope; EDXS – energy dispersive X-ray spectroscopy in a field-emission-gun scanning electron microscope.

and then specimens were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). After washing with 0.1 M cacodylate buffer, the samples were postfixed in 1% osmium tetroxide for 2 h, washed again and dehydrated in an ascending series of ethanol and in absolute acetone. Samples were embedded in Agar 100 resin. Prior to embedding, mancae were perforated with a thin needle for better infiltration of resin. Polymerization of the resin was performed at 60 °C for 48 h in embedding molds. In addition, acrylic resin LR White embedded specimens that had been formerly fixed in 2% paraformaldehyde and 0.25% glutaraldehyde in 0.1 M Hepes buffer (pH 7.2) and non-osmicated, and Agar 100 resin embedded specimens that had been formerly fixed in methanol, were also included in ultrastructural analysis. Semithin sections were cut with a glass knife, stained with Azur II-Methylene Blue and imaged with a Zeiss AxioImager Z.1 light microscope, equipped with an HRC Axiocam camera using Axiovision software. Ultrathin sections were cut by a Reichert Ultracut S ultramicrotome (Leica) with a diamond knife and contrasted with 4% uranyl acetate and 10% lead citrate. They were inspected and recorded with a Philips CM100 transmission electron microscope, equipped with the cameras BioScan 792 and Orius 200 (Gatan) using Digital Micrograph software.

2.3. Labelling with WGA lectin

Labelling with lectin wheat germ (*Triticum vulgare*) agglutinin (WGA) was performed on two stages of late embryos, on three stages of marsupial mancae (Table 1) and on tergites from an adult animal. Specimens destined for labelling with WGA lectin were processed for LR White embedding as described in Section 2.2. Ultrathin sections were incubated in 1% BSA (bovine serum albumin, Sigma) in 0.1 M Hepes for 5 min, followed by incubation in lectin wheat germ agglutinin (WGA) conjugated to 10-nm gold (Gentaur) for one hour at room temperature. After washing in 0.1 M Hepes, the sections were fixed in 1.25% glutaraldehyde in 0.1 M Hepes for 30 min at room temperature and washed in 0.1 M Hepes then in deionised water. Samples of adult *P. scaber* decalcified cuticle were used as a positive control for chitin localization and were always labelled simultaneously with the samples of embryos and mancae. Other tissues in the section, e.g. muscles and yolk, were considered as negative controls. Sections were inspected with a Philips CM100 transmission electron microscope, equipped with BioScan 792 (Gatan) camera using Digital Micrograph software.

2.4. Energy dispersive X-ray spectroscopy (EDXS)

Energy dispersive X-ray spectroscopy (EDXS) in field-emission-gun scanning electron microscopy (FEG-SEM) was performed to determine the elemental composition of the marsupial mancae cuticle. Tergites of three adult animals and early marsupial mancae in two sequential stages were analysed (Table 1). Adult tergites and mancae were fixed in absolute methanol then air-dried. Some of the samples were fractured to expose internal transverse or longitudinal views. Mounted specimens were coated with an approximately 5-nm carbon layer to ensure electrical conductivity of the specimens in the FEG-SEM. EDXS analyses were performed in a high-resolution field-emission-gun scanning electron microscope (JEOL JSM-7600F) equipped with an Oxford Instruments INCA system for energy dispersive X-ray spectroscopy. An acceleration voltage of 15 kV and a working distance of 15 mm were used for the EDXS measurements. Each X-ray spectrum was collected for 180 s using INCA software.

3. Results

3.1. Ultrastructure of cuticle in embryos and larvae

To study cuticle formation during *P. scaber* development, we first examined the ultrastructure of the apical epidermal matrices in several sequential stages of development in marsupium. Early signs of epidermal matrix deposition are evident in the mid-stage embryo at stage 10 (56% of embryonic development) (Fig. 1A–D). The apical plasma membrane of epidermal cells forms distinctive bulges with electron dense tips and a delicate sheet of material spreads over the surface of epithelium (Fig. 1C and D). In the next examined stage 13 (68% of embryonic development) (Fig. 1E) a continuous electron dense lamina is evident on the surface of epidermis (Fig. 1F–H). The lamina mostly follows the outline of the apical plasma membrane, but distinguishably from cuticular matrix it does not match precisely the contour of the cell membrane in some regions. The electron dense lamina is about 15 nm thick and does not show any discernible substructure. The narrow space between the lamina and the apical plasma membrane contains loosely and irregularly arranged material. Electron dense material accumulations on the tips of the plasma membrane bulges are apparent (Fig. 1H). The lamina and subjacent material appear to represent the early precuticular matrix.

The next two stages, stage 14 and stage 15 (72–76% of embryonic development), are very similar to each other regarding the ultrastructure of the extracellular matrices covering the embryonic epidermis (Fig. 2). Precuticular matrix composed of the electron dense lamina and the subjacent loose material is evident on the embryo surface (Fig. 2B and C). In addition to this, another electron dense lamina and a thin layer of forming matrix are closely apposed to the apical plasma membrane (Fig. 2C–E). Conspicuous secretion of the newly forming matrix is evident as a fibrous material spreading from the tips of the plasma membrane bulges (Fig. 2D). The two electron dense laminae are similar in structure and in thickness. In favourable cross sections a trilayered appearance of laminae is evident, with electron dense layers enclosing an electron lucent layer in between. Trilayered structure is more distinct in the lamina of the forming precuticular matrix just above the plasma membrane (Fig. 2E).

In late embryo of stage 16 (80% of embryonic development) (Fig. 3A and B) a substantial precuticular matrix underneath the vitelline membrane is present (Fig. 3C–E). This matrix varies in thickness from 0.05 μm to 0.7 μm in the same specimen and often displays a wavy surface (Fig. 3C–E). It consists of moderately dense material, which does not display any prominent organization pattern, and an overlying electron dense lamina. In some places clumps of dense grainy material are observed within the matrix. Similarly to previous stages, three layers of the dense lamina are discernible in cross sections, exposing an electron lucent layer between the dense layers. The apical plasma membrane of the epidermal cells is slightly rough, forming evenly arranged bulges with electron dense tips (Fig. 3C). Electron dense vesicles in the apical cytoplasm are occasionally observed in this stage (Fig. 3E). In addition to this precuticular matrix, intense foldings of electron dense lamina are observed underneath the vitelline membrane in some specimens (Fig. 3F–H). Folded lamina in these stacks is also observed to be trilayered (Fig. 3H) and we consider that these piled sheaths correspond to the shed electron dense lamina and the associated material of the previous precuticular matrix.

The prehatching late embryo of stage 18 (88–96% of embryonic development) is tightly enclosed by the vitelline membrane (Fig. 4A and B). This is the earliest stage in which cuticle

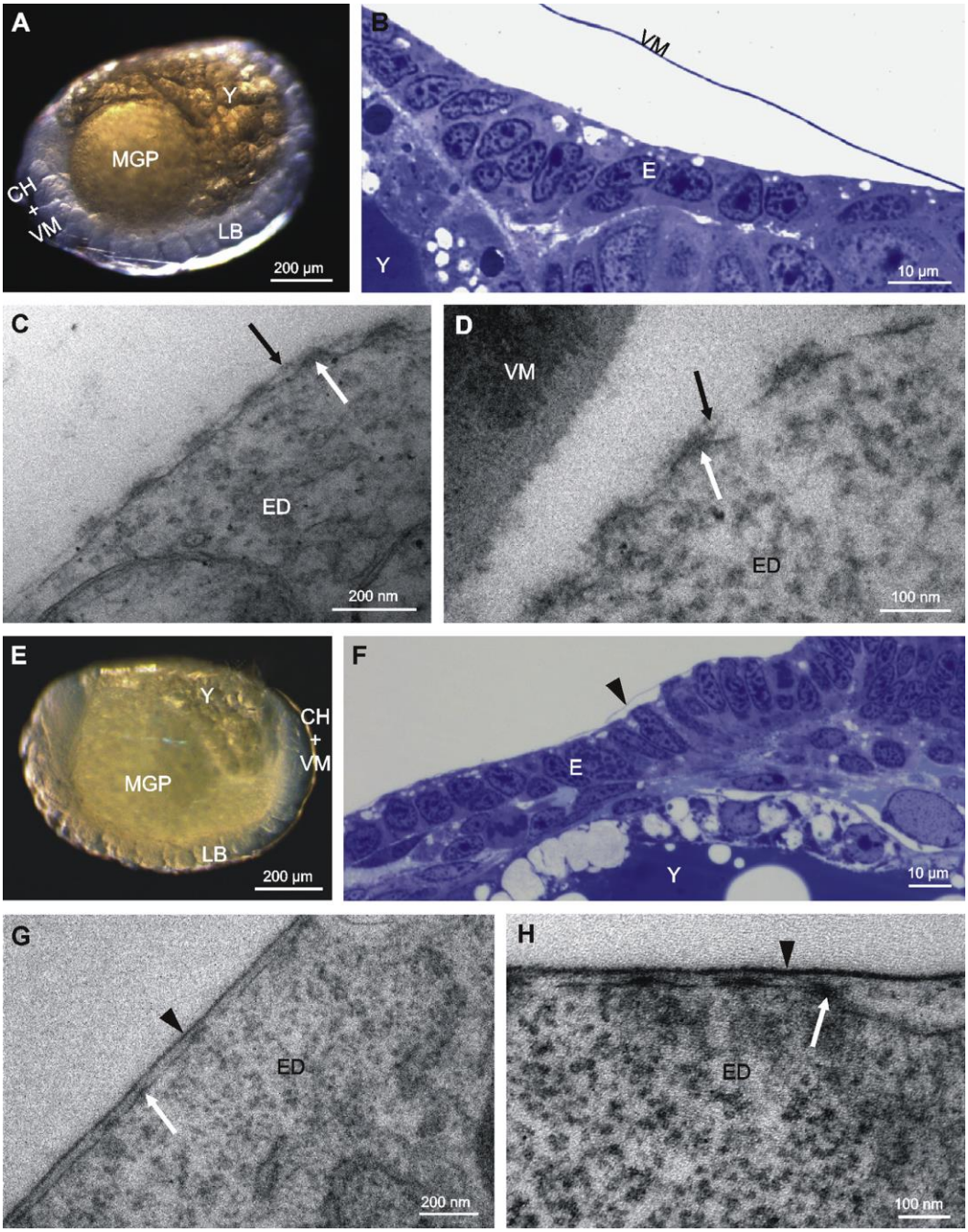


Fig. 1. A–D. Mid-stage embryo (stage 10). A. Embryo with limb buds and midgut glands primordia. B. Semithin section of the epidermis, covered by the vitelline membrane. C, D. Ultrastructure of the epidermal cell apical region and extracellular matrix. Plasma membrane bulges with electron dense tips (white arrow) and overspread delicate sheet of material (black arrow). E–H. Mid-stage embryo (stage 13). E. Embryo with caudally extended midgut glands enclosing the great part of yolk. F. Semithin section of the epidermis. A thin lamina of the early precuticular matrix is apparent (arrowhead). The vitelline membrane is still present but not seen in the image frame. G, H. Ultrastructure of the epidermal cell apical region and extracellular matrix. The early precuticular matrix consists of an electron dense lamina (arrowhead) and subjacent loosely arranged material. On the tips of the plasma membrane bulges (white arrow) electron dense material accumulations are evident. Chorion (CH), epidermis (E), epidermal cell (ED), limb buds (LB), midgut glands primordia (MGP), vitelline membrane (VM), unenclosed yolk (Y).

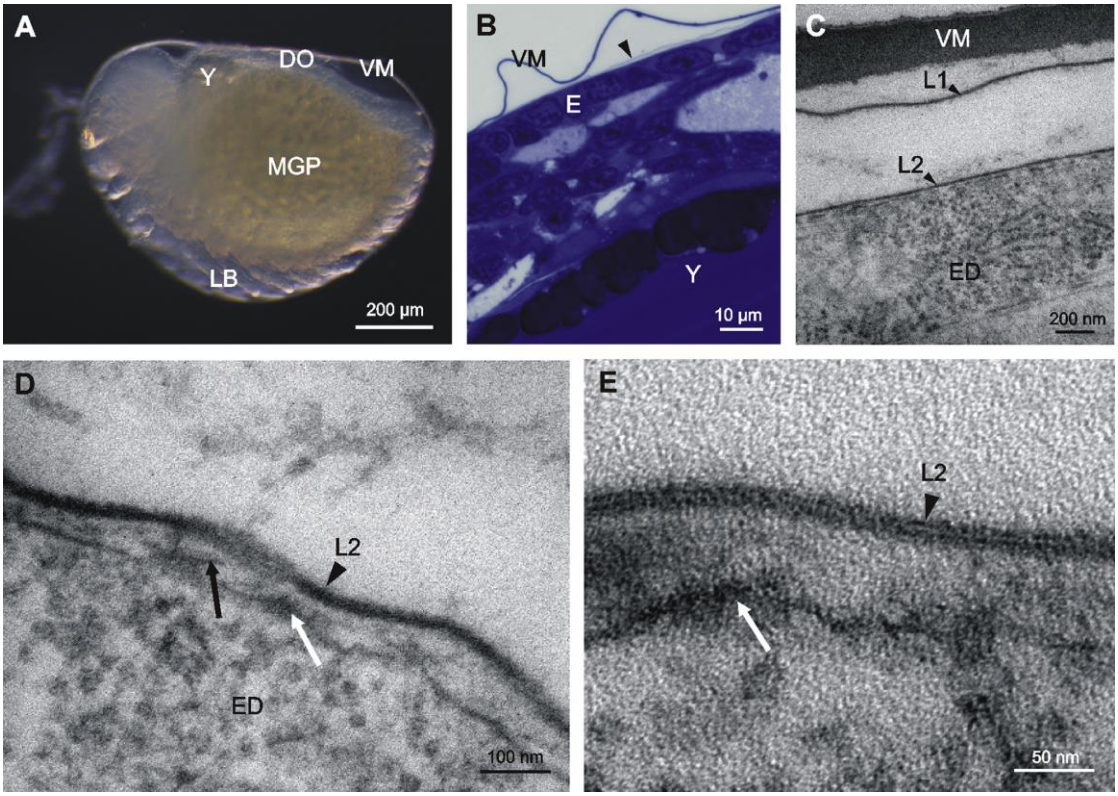


Fig. 2. Mid-stage embryo (stages 14 and 15). A. Chorion was removed artificially and the embryo is surrounded only by vitelline membrane. A saddle-shaped dorsal organ is evident. The majority of yolk is enclosed within the midgut glands. B. Semithin section of the embryo. The vitelline membrane and a thin lamina of the precuticular matrix (arrowhead) are visible above the epidermis. C. The precuticular matrix consisting of electron dense lamina (L1) and a small amount of loose material covers the epidermal cell. Another lamina (L2) closely apposed to apical plasma membrane is evident. D. Secretion of the newly forming precuticular matrix (black arrow) is apparent on the apical plasma membrane bulges (white arrow). E. The lamina of the forming precuticular matrix (L2) displays trilayered appearance. Dorsal organ (DO), epidermis (E), epidermal cell (ED), limb buds (LB), midgut glands primordia (MGP), vitelline membrane (VM), unenclosed yolk (Y).

formation is evident. The newly forming cuticle is closely apposed to the apical plasma membrane of epidermal cells and is strictly aligned to the ruffled cell surface (Fig. 4C). The cuticle thickness is approximately 0.10 µm and it is uniform in all embryo sections examined. In the new cuticle the electron dense epicuticle is clearly resolved and underneath it the forming procuticle is visible (Fig. 4D–F). The epicuticle shows several thin layers with different electron densities (Fig. 4E). Epicuticular scales are formed on the apices of cell ruffles and extend into the precuticular matrix (Fig. 4C). The apical plasma membrane forms bulges with electron dense tips (Fig. 4D and E). The oblique sections of the epidermal cell surface show that the rounded plasma membrane bulges are evenly arranged and the fibrous material extends around their electron dense tips (Fig. 4F). Above the forming cuticle the precuticular matrix is still present and its structure is generally similar to that in stage 16. In some regions the grainy material within the matrix is more abundant compared to earlier stages, while in others lucent spaces above the newly forming cuticle are present (Fig. 4C–F). The next stage of prehatching embryo (stage 19), which is only loosely covered by the vitelline membrane, the cuticle is thicker (about 1–3 µm) and differentiated into distinct layers (Fig. 4G and I). The procuticle is subdivided into exo- and endocuticle, which display several sublayers (Fig. 4G–I). In some regions cuticle detachment from epidermis, disintegration of the inner endocuticular layers and perforation of the cuticle by pore canals are observed (Fig. 4H and I).

In newly hatched early marsupial manca (Fig. 5A) the epidermis is covered with a cuticle (Fig. 5B), aligned to the ruffled surfaces of epidermal cells. This cuticle is up to 1 µm thick and exhibits layered structure (Fig. 5C–F). Epicuticle forms distinct cuticular scales (Fig. 5C) and morphologically resembles the epicuticle of adults (Fig. 5D). The procuticle is the major part of the cuticle and the chitin–protein fibres arrangement is discernible, especially in non-osmicated specimens and in methanol-fixed specimens (Fig. 5E and F). Broad cytoplasmic projections that extend into the procuticle are frequently evident (Fig. 5D). In one specimen an additional sheath above this cuticle was observed in some regions (Fig. 5G and H). This sheath consists of the outer layer resembling the epicuticle, which encloses lucent material (Fig. 5H). We presume that this sheath represents the remnants of the degraded preceding cuticle, composed of epicuticle and disintegrated procuticle.

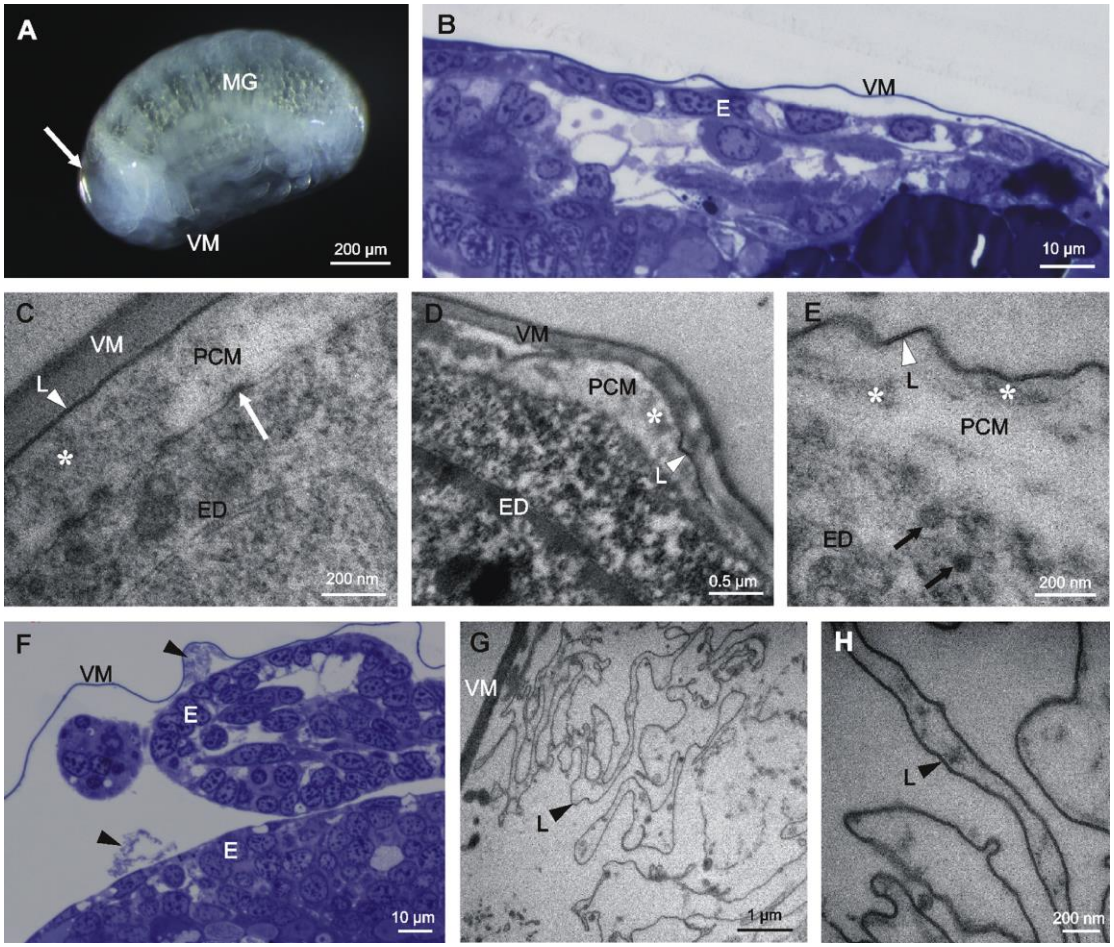


Fig. 3. Late embryo (stage 16). A. Embryo is ventrally bent and covered only by the vitelline membrane. Pigmented eye is evident (arrow). Yolk is completely enclosed within the midgut glands. B. Semithin section of the epidermis. C–E. The precuticular matrix is substantial and consists of an electron dense lamina (white arrowheads) and subjacent matrix with no visible organization pattern, containing clumps of dense grainy material (white *). C. The apical plasma membrane forms bulges with electron dense tips (white arrow). E. Apical cytoplasm contains electron dense vesicles (black arrows). F–H. Piles of folded trilayered lamina (black arrowheads) of the shed precuticular matrix underneath the vitelline membrane. Epidermis (E), epidermal cell (ED), lamina (L), midgut glands (MG), precuticular matrix (PCM), vitelline membrane (VM).

The exoskeletal cuticle of advanced early and mid-stage marsupial mancae (Fig. 6A) already has the main structural features of the adult crustacean cuticle but it is significantly thinner (1–2 µm thick) than adult cuticle (Fig. 6B–E). Epicuticle structure and thickness are similar to those in the newly hatched early marsupial manca. Procuticle shows distinct exocuticle and endocuticle with pronounced differentiation in lamellar sublayers (Fig. 6C–E). In some specimens bulges with electron dense tips or cytoplasmic projections are evident on the apical plasma membrane (Fig. 6C and E).

In the cuticle of late marsupial manca (Fig. 6F) a distinct arrangement of chitin–protein fibres is apparent in both exo- and endocuticle (Fig. 6H–J). In this stage the renewal of the cuticle is observed. The cuticle is detached from the epidermis and the ecdysial space is formed (Fig. 6G and J). Endocuticle of the detached exoskeleton is not uniform as the inner layers are more electron dense (Fig. 6J). In several specimens a newly forming cuticle is evident over the ruffled surfaces of epidermal cells (Fig. 6J). The new cuticle is 150–200 nm thick in this stage and consists of a thin electron dense epicuticle and electron lucent procuticle. In some regions the procuticle appears homogenous, while in some regions a network of chitin–protein fibres is visible (Fig. 6J–L). The apical plasma membrane forms regularly arranged bulges (Fig. 6K). In the posterior region of the animal broad cytoplasmic projections invading the new procuticle are observed, similarly as in the cuticle of newly hatched marsupial manca (Fig. 6L).

3.2. WGA lectin–gold labelling

To obtain the information on the composition of the organic scaffold in the apical epidermal matrices, formed during *P. scaber*

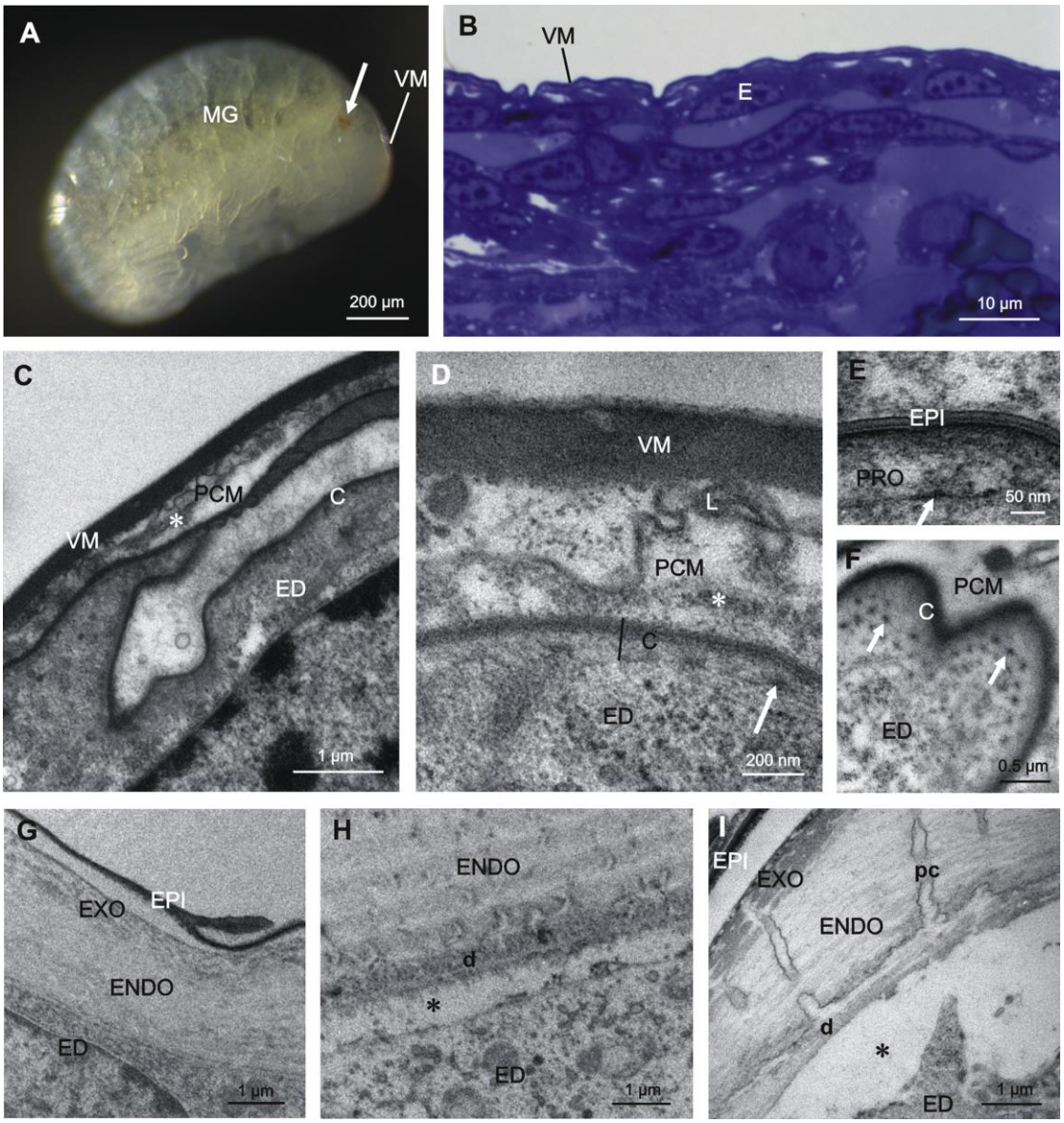


Fig. 4. A–F. Prehatching late embryo (stage 18). A. Embryo is larger and tightly enclosed by the vitelline membrane. Pigmented eye is evident (arrow). B. Semithin section of the embryo peripheral region. Epidermis is tightly covered by the vitelline membrane. C–F. The exoskeletal cuticle is formed above the epidermal cell, strictly aligned to the ruffled cell surface and consists of electron dense epicuticle and forming procuticle. C, D. The precuticular matrix is present above the cuticle and contains abundant grainy material (white *) under the wavy dense lamina. C. Forming epicuticular scale extends into the precuticular matrix. E. The epicuticle shows several thin layers with different electron densities. D, E. Apical plasma membrane forms bulges with electron dense tips (white arrows). F. Membrane bulges, observed in oblique sectioned epidermal cell surface, are evenly arranged, with fibrous material around their tips (white arrows). G–I. Prehatching late embryo (stage 19), in which the vitelline membrane was removed artificially. The cuticle is thick and differentiated in epicuticle, exocuticle and endocuticle. H, I. In some regions cuticle detachment from epidermis (black *) and endocuticle disintegration (d) are evident. Cuticle (C), endocuticle (ENDO), epidermis (E), epidermal cell (ED), epicuticle (EPI), exocuticle (EXO), lamina (L), midgut glands (MG), pore canals (pc), precuticular matrix (PCM), procuticle (PRO), epicuticular scale (SC), vitelline membrane (VM).

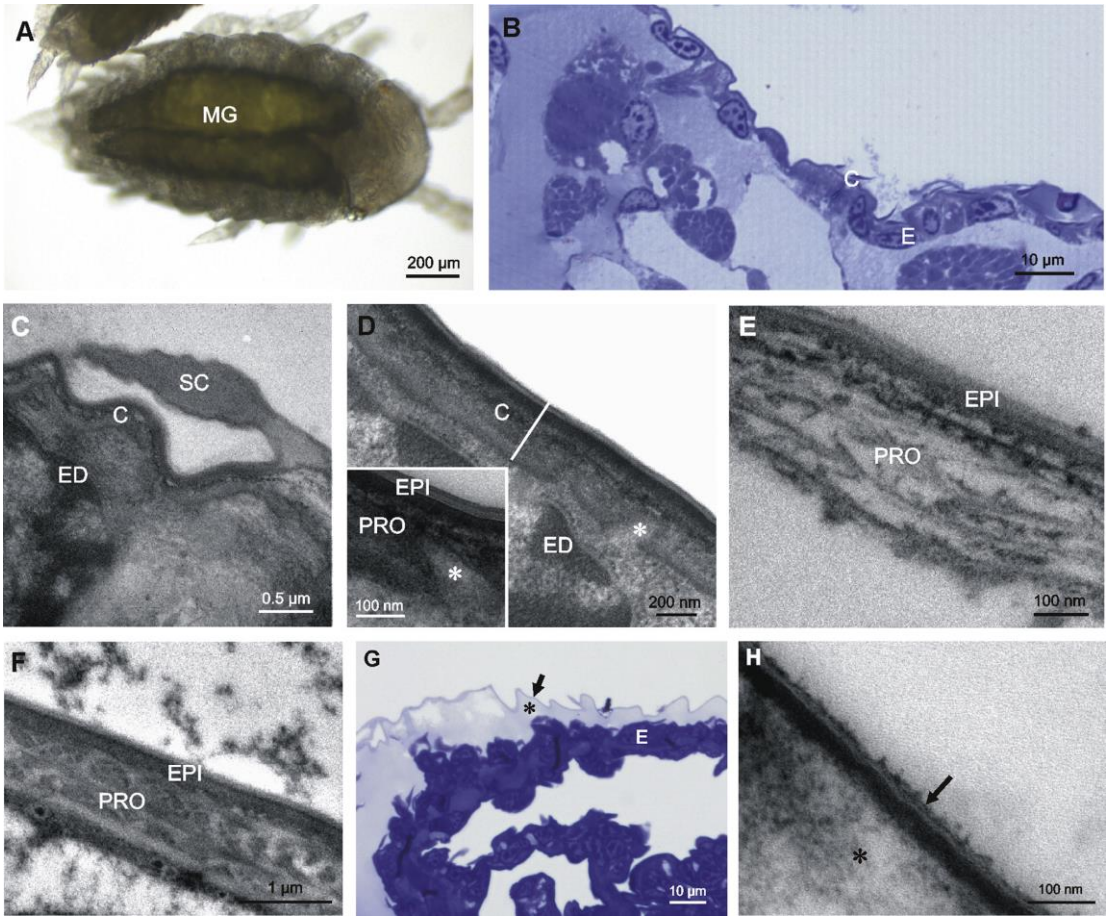


Fig. 5. Newly hatched early marsupial manca. A. Manca is 1.4–1.5 mm long and has large midgut gland tubes. B. Epidermis is covered by a thin cuticle with distinct cuticular scales. C–F. The exoskeletal cuticle consists of epicuticle and procuticle. D. The procuticle is invaded by broad cytoplasmic projections (white *). D insert. In higher magnification the differentiated structure of the epicuticle is evident. E, F. Chitin–protein fibres arrangement of the procuticle in (E) methanol-fixed specimen and in (F) non-osmicated, aceton-free dehydrated specimen. G, H. The remnants of the degraded preceding cuticle (black *) are observed in some regions of the manca surface in one specimen. The outer layer resembles the epicuticle (black arrow). Cuticle (C), epidermis (E), epidermal cell (ED), epicuticle (EPI), midgut glands (MG), procuticle (PRO), epicuticular scale (SC).

intramarsupial development, we performed localization of *N*-acetyl-glucosamine oligomers, including chitin, by WGA lectin–gold labelling in five different developmental stages: in two stages of late embryo, in two stages of early marsupial manca and in late marsupial manca. Tergites of adult animals were labelled and used as a reference.

The precuticular epidermal matrix in late embryos of stage 16 displays no labelling by WGA lectin–gold (Fig. 7A). In the stage 18 late embryos, a sparse binding of WGA lectin–gold is observed in precuticular matrix, particularly in denser regions of this matrix. Additionally, labelling is evident in the thin newly forming cuticle in stage 18 late embryos (Fig. 7B). More conspicuous labelling is observed in the cuticle of newly hatched early marsupial mancae (Fig. 7C). Very intense labelling is apparent in the thicker cuticle of advanced early marsupial mancae (Fig. 7D) and resembles the WGA lectin–gold labelling of the cuticle of adults (Fig. 7F). In late marsupial mancae, where cuticle renewal is evident, the detached cuticle is densely labelled and binding of WGA lectin–gold is also observed in the new cuticle apposed to the apical surface of epidermal cells (Fig. 7E).

3.3. Elemental composition of the cuticle

To examine if exoskeleton calcification starts during intramarsupial development in *P. scaber*, we performed EDXS analyses of the exoskeletal cuticle in two sequential stages of early marsupial mancae in comparison to tergites of adult animals.

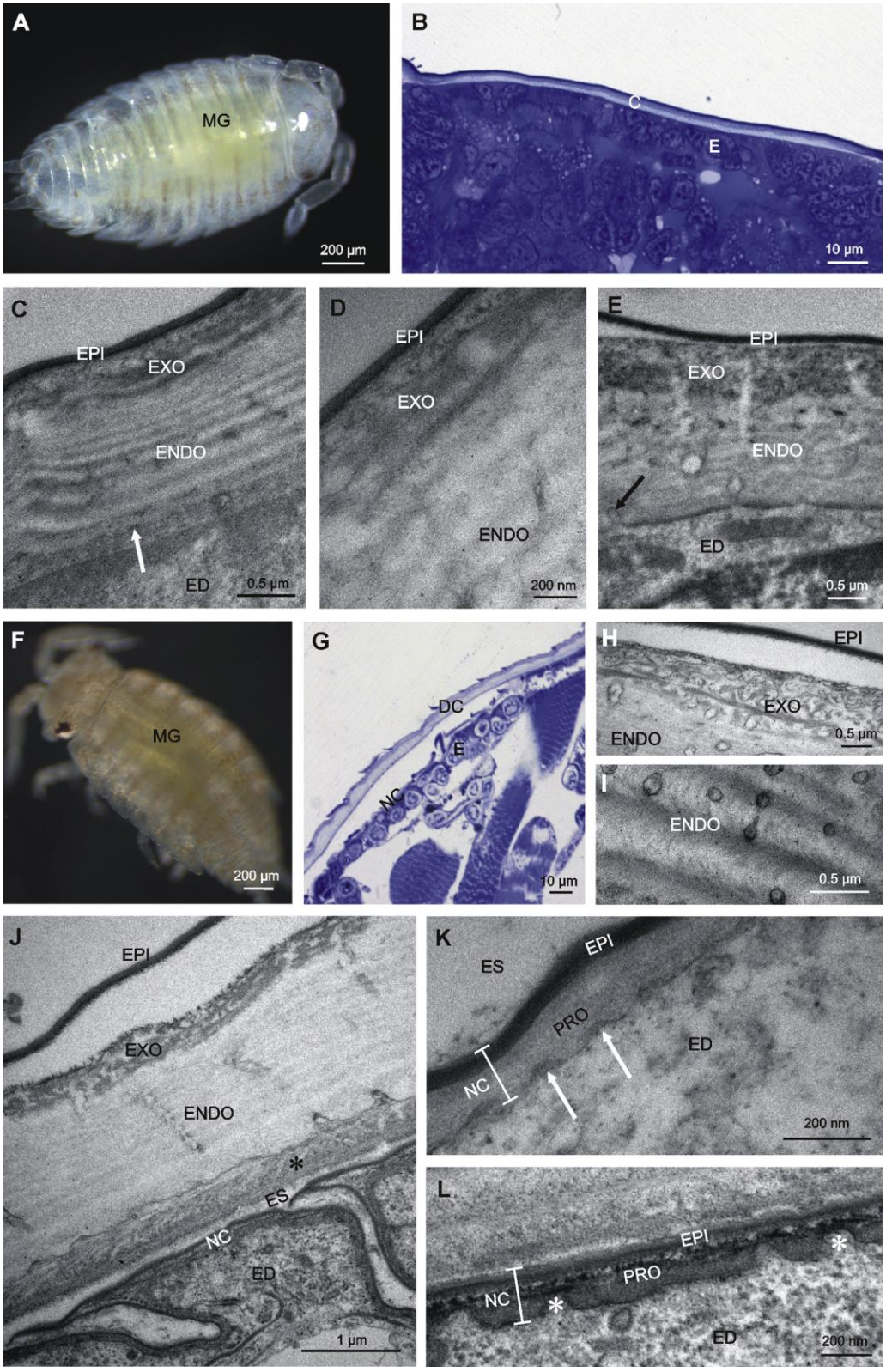
The energy dispersive X-ray spectra obtained from the surface and from the transverse fractures of tergite cuticle in adults show prominent contents of calcium. Additional principal elements detected are phosphorus, sulphur, magnesium, oxygen and carbon (Fig. 8). Calcium peaks are several times higher compared to phosphorus peaks. In advanced early-stage

marsupial mancae, the spectra obtained from the surface and from the transversely fractured tergite cuticle also show conspicuous calcium peaks that are evidently higher than phosphorus peaks. Magnesium, sulphur, oxygen and carbon are also detected in the cuticle (Fig. 9). In newly hatched early marsupial mancae the spectra obtained from the cuticle reveal low calcium peaks in addition to phosphorus, magnesium, sulphur, carbon and oxygen peaks (Fig. 10). The ratio of calcium to phosphorus peaks is always below 1 in the specimens of this stage. Spectra of the internal tissues do not display calcium, magnesium and phosphorus peaks.

4. Discussion

In this study formation of the exoskeletal cuticle during intramarsupial embryonic and larval development of the terrestrial isopod crustacean *P. scaber* was investigated. Our ultrastructural analysis reveals that in the second half of embryonic development in the isopod *P. scaber*, at least two precuticular matrices are formed by the embryonic epidermal cells prior to cuticle formation. The entire surface of embryos in all stages examined was analysed and no differences were observed in the precuticular matrices ultrastructure between different body locations. Early signs of epidermal matrix formation are observed in mid-stage embryos. The early precuticular matrix deposition takes place underneath the vitelline membrane on the tips of apical plasma membrane bulges, and is initially observed as a fine, delicate sheet and later as a substantial lamina with subjacent loosely arranged material. Subsequently in the mid-stage embryo another precuticular matrix is deposited beneath the previous one. The electron dense laminae are structurally similar in both precuticular matrices, while the underlying material is more compact and dense in the later precuticular matrix compared to the early one. The early precuticular matrix is soon shed, which is evidenced by the piled sheaths underneath the vitelline membrane. Shedding of the early precuticular matrix and deposition of the next precuticular matrix occur during the transition from mid-stage embryo to late-stage embryo. In this period, the embryo displays major external morphological changes such as prolongation and ventral bending of the body to attain a comma-like structure. At this point of development the embryo also hatches from the chorion, rotates in the dorso-ventral axis within the vitelline membrane and loosens its connection with the extraembryonic dorsal organ (Milatovič et al., 2010). The dorsal organ presumably plays an osmoregulatory role, as proposed by Meschenmoser (1989) and thus loosening of the connection between the dorsal organ and embryo is likely to be related to changes in the osmoregulatory capacity of the embryo. In peracarid crustaceans with intramarsupial embryonic development, the embryonic epidermal matrices preceding cuticle formation were ultrastructurally described in the aquatic amphipod *P. hawaiensis* (Havemann et al., 2008) and in the isopod *H. balani* (Goudeau, 1976). In arthropods, altogether two to five epidermal matrices are secreted by the embryo and the final epidermal matrix is the larval cuticle. The embryonic epidermal matrices observed before cuticle formation are termed: (i) embryonic cuticles (Dorn, 1976; Belk, 1987; Konopova and Zrzavy, 2005; Moussian et al., 2006; Havemann et al., 2008), (ii) embryonic membranes (Ziese and Dorn, 2003) or (iii) embryonic envelopes (Goudeau, 1976; Goudeau and Lachaise, 1983; Glas et al., 1997). The cryptobiotic cyst of the branchipod *A. salina* features one 'embryonic cuticle' that displays several structural features found in the exoskeleton of adult crustaceans. During further development a larval exoskeleton is formed underneath the 'embryonic cuticle' before hatching (Morris and Afzelius, 1967). In the decapod *P. pugio*, the fifth matrix that is secreted by the embryonic epidermis finally shows cuticular appearance (Glas et al., 1997). As reported in the literature, the 'embryonic cuticles'/'embryonic envelopes' are degraded and shed and the first exoskeletal cuticle that persists through the early larval development is already formed before hatching. In our study of isopod *P. scaber* a structural resemblance of the two precuticular matrices to the 'embryonic cuticles' of other arthropods is evident. With respect to the origin and function of the precuticular matrices in other arthropods, several explanations have been presented. It is most frequently proposed that the precuticular matrices are merely vestigial, somewhat reduced cuticles (Morris and Afzelius, 1967; Ziese and Dorn, 2003; Konopova and Zrzavy, 2005; Moussian et al., 2006). The electron dense laminae of the precuticular matrices resemble the trilayered structure of the insect cuticle envelope, previously termed cuticulin layer, which is the first layer of the new cuticle to be deposited at molting (Locke, 2001). This resemblance was observed also in other studies (Ziese and Dorn, 2003; Konopova and Zrzavy, 2005; Havemann et al., 2008). Nevertheless, the embryonic precuticular matrices may participate in protection of the embryo, as also suggested by Goudeau (1976), Glas et al. (1997) and Ziese and Dorn (2003). We presume that the precuticular matrices may also control the transport of materials through the embryo surface and establish an adequate environment for cuticle formation.

The ultrastructure of the cuticles was analysed in the dorsal side of the body (tergites). The matrix that displays typical characteristics of crustacean exoskeletal cuticle starts to form in the prehatching embryo underneath the subsistent precuticular matrix. The epicuticle of the newly formed cuticle has in this stage similar five-layered structure as the outer epicuticle of adult *P. scaber* (Ziegler, 1997). During progression of embryonic development, cuticle thickening and structural differentiation are evident. In the last stage of embryonic development (prehatching embryo stage 19) the cuticle is differentiated in epicuticle and exo- and endocuticle with distinct sublayers. Cuticle detachment and degradation of endocuticle are observed. Morphological features of cuticle renewal, i.e. the cuticle detachment and degradation in prehatching embryo stage 19 and the new cuticle formation in newly hatched manca, show that the first molting occurs approximately in the same period as hatching from vitelline membrane. Absence of the egg envelopes after hatching results in direct exposure of the manca's surface to the marsupial fluid and enables extension of the body and relaxation of the appendages. In newly hatched manca, formation of the new relatively thin cuticle, that already displays differentiated structure of epi- and procuticle, is evident. In further development of marsupial manca (advanced early manca and mid-stage manca) the procuticle thickens and displays pronounced differentiation of exo- and endocuticle, structurally similar to the cuticle of adults. In some specimens the apical plasma membrane bulges with electron dense tips and cytoplasmic projections into the cuticle are evident. The exoskeletal cuticle in the marsupial manca serves to protect and support the animal in the aqueous environment, while keeping the body and appendages flexible and capable of extension and movement. In late marsupial manca the cuticle detaches from the epidermis (apolysis) and displays inner layers with higher electron density, indicating initial disintegration. The ecdysial


A
MG
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space is evident and in several specimens a new cuticle was formed. Our results suggest that exuviation of manca occurs shortly after it emerges from marsupium. Henceforth the manca is exposed to the external environment and becomes an independent and active individual, which requires the protection and support of the larval body by the fully formed surface cuticle.

Plasma membrane modifications are observed in many stages examined in our study. Flat plasma membrane bulges with electron dense tips are evident in the stages with precuticular matrix and in the stages with thin newly forming cuticle covering the epidermis. Broad cytoplasmic projections extend into the new cuticle of newly hatched marsupial manca and late marsupial manca. According to the literature, both modifications indicate that synthesis of a precuticular or a cuticular matrix is still in progress. Similar surface modifications of the epithelial cells were observed during active cuticle synthesis in many studies: plasma membrane protrusions (microvilli-like structures) of various lengths (Locke, 1961; Locke and Huie, 1979; Koulish and Klepal, 1981; Compere, 1995; Ziegler, 1997; Elliot and Dillaman, 1999; Locke, 2001; Moussian et al., 2006; Havemann et al., 2008; Vittori et al., 2012) and cytoplasmic extensions within pore canals in the cuticle (Locke, 1961; Compere and Goffinet, 1987b; Compere, 1990). Several authors associate these structures with secretion of different cuticular components (Locke, 1961; Locke and Huie, 1979; Compere and Goffinet, 1987b; Compere, 1990). We consider that electron dense tips of the plasma membrane bulges described in our study correspond to the plaques, the sites of the initial polymerization and organization of the developing cuticular components (Locke and Huie, 1979; Locke, 2001; Moussian et al., 2005; Moussian, 2010), observed also in adult molting crustaceans (Koulish and Klepal, 1981; Compere, 1995; Ziegler, 1997). The terminology and function of the plasma membrane of cuticle producing epithelial cells are discussed in Moussian (2013). Our study also shows that the surfaces of epidermal cells are ruffled during the stages when the new cuticle is formed. This enables growth of embryos and mancae during development, as the forming cuticle, adopting the shape of the ruffled epidermal cells surfaces, presents a larger surface than the old cuticle.

To obtain initial information on the composition of organic framework constituting precuticular matrix in embryos and cuticle in marsupial mancae, we performed labelling with wheat germ agglutinin lectin. WGA lectin binds highly specifically to *N*-acetyl-D-glucosamine and has a strong affinity to oligomers and polymers of *N*-acetyl-glucosamine, especially to chitin (Allen et al., 1973; Peters and Latka, 1986). Therefore it has frequently been used as a chitin-localization probe (e.g. Shillito et al., 1995; Neuhaus et al., 1997; Lemburg, 1998). The combining site of wheat germ agglutinin was reported to be complementary to a sequence of three β-(1 → 4)-linked *N*-acetyl-glucosamine residues (Debray et al., 1981), but interactions of WGA lectin with *N*-acetylneuraminic acid containing glycoconjugates (Monsigny et al., 1980) and poly-*N*-acetyllactosaminoglycans (Gallagher et al., 1985) were shown to occur. A significance of ligand's configuration and linkage type for the specificity of lectin binding (Iskratsch et al., 2009) and the existence of eight simultaneously functional sugar binding sites on the WGA dimer (Schwefel et al., 2010) indicate in addition a complexity of WGA interactions with cell surface glycoconjugates. Thus, careful interpretation of WGA binding is needed and in the case of chitin localization, additional aspects require attention (e.g. chitin in the form of chitin–protein fibres) and additional data such as ultrastructural or chemical analyses are beneficial (Schmidt et al., 1998; Steinbrecht and Stankiewicz, 1999; Martin et al., 2006; Moussian et al., 2006; Havemann et al., 2008). The chitin-based cuticle in adult specimens of *P. scaber* displays intense labelling with WGA lectin–gold complex (Fig. 7F). Binding of the WGA lectin gold probes to the cuticle in marsupial mancae and to the thin newly forming cuticles in molting marsupial mancae and in late embryos of the stage 18 suggests that the organic scaffold of these cuticles resembles the adult cuticle. In addition, the ultrastructure of these cuticles exhibits the main features of the adult crustacean cuticle. Our results suggest that the precuticular matrix differs from cuticle in adults and mancae regarding the organic scaffold. The sparse labelling observed in some regions of the precuticular matrix in stage 18 embryos indicates the presence of WGA binding glycoconjugates or saccharides. The precuticular matrix with a wavy surface also is ultrastructurally different from chitin-based cuticles of adults and mancae. Our results are in agreement with results of WGA-gold labelling in the study of *Drosophila* embryo cuticle differentiation (Moussian et al., 2006). The precuticular matrix, termed an embryonic cuticle, is not based on a chitin organic component, while chitin is present in the procuticle of subsequent larval cuticle. In the study of amphipod crustacean *P. hawaiensis* a detection of chitin with WGA labelling in the embryonic cuticle has been reported (Havemann et al., 2008).

The elemental composition of the cuticle during intramarsupial development has not been reported before. Elemental and mineral distributions in the cuticle have been investigated in adult isopod crustaceans (Štrus and Compere, 1996; Hild et al., 2008, 2009; Neues et al., 2011; Seidl et al., 2011; Ruangchai et al., 2013). We show here that in newly hatched early marsupial mancae, a conspicuous calcium peak is detected in the exoskeletal cuticle. In addition, phosphorus and magnesium are evident in EDXS spectra obtained from the larval surface. The ratio of calcium to phosphorus peaks is below 1 in all spectra, which is in contrast to typically high calcium to phosphorus ratios in the adult cuticles. Our analysis suggests that calcium sequestration occurs in the exoskeletal cuticle of newly hatched marsupial mancae and that the mineral composition of this matrix is different from that in adults. Our next observation here is that the elemental composition, including ratios between elemental peaks, in advanced early stage marsupial mancae exoskeletal cuticle is similar to that in adults. Prominent calcium peaks are evident in EDXS spectra, which also display magnesium, phosphorus, carbon, oxygen and sulphur content. In advanced early mancae and in adults, calcium peaks are several times higher than phosphorus peaks. Similar elemental

Fig. 6. A–E. Advanced early marsupial manca and mid-stage marsupial manca. A. Manca is around 1.6 mm long. Integument pigmentation is evident. B. Semithin section: The epidermis is covered by a cuticle. C–E. Ultrastructure of the exoskeletal cuticle, consisting of epicuticle, exocuticle and endocuticle. D. Exo- and endocuticle display lamellar sublayers of chitin–protein fibres. C. Bulges with electron dense tips (white arrow) and E. cytoplasmic projections (black arrow) are formed on the apical plasma membrane of the epidermal cells. F–L. Late marsupial manca. F. Manca is around 1.9 mm long, with pronounced locomotion and reduced yolk in the midgut glands. G. Semithin section: Exoskeleton renewal is evident – cuticle detachment from the epidermis and a new cuticle formation. H–L. Ultrastructure of the exoskeleton. H–J. In exocuticle and endocuticle of the detached exoskeleton sublayers of chitin–protein fibres arrangement are distinct. J. The electron denser inner layers of the endocuticle indicate disintegration (black *). The newly forming cuticle is aligned with the ruffled cell surface. K, L. The new cuticle consists of epicuticle and procuticle. K. Apical plasma membrane forms bulges (white arrows). L. The cytoplasmic projections invading the new cuticle were observed in the posterior region of one specimen (white *). Cuticle (C), detached cuticle (DC), ecdysial space (ES), endocuticle (ENDO), epidermis (E), epidermal cell (ED), epicuticle (EPI), exocuticle (EXO), midgut glands (MG), new cuticle (NC), procuticle (PRO).

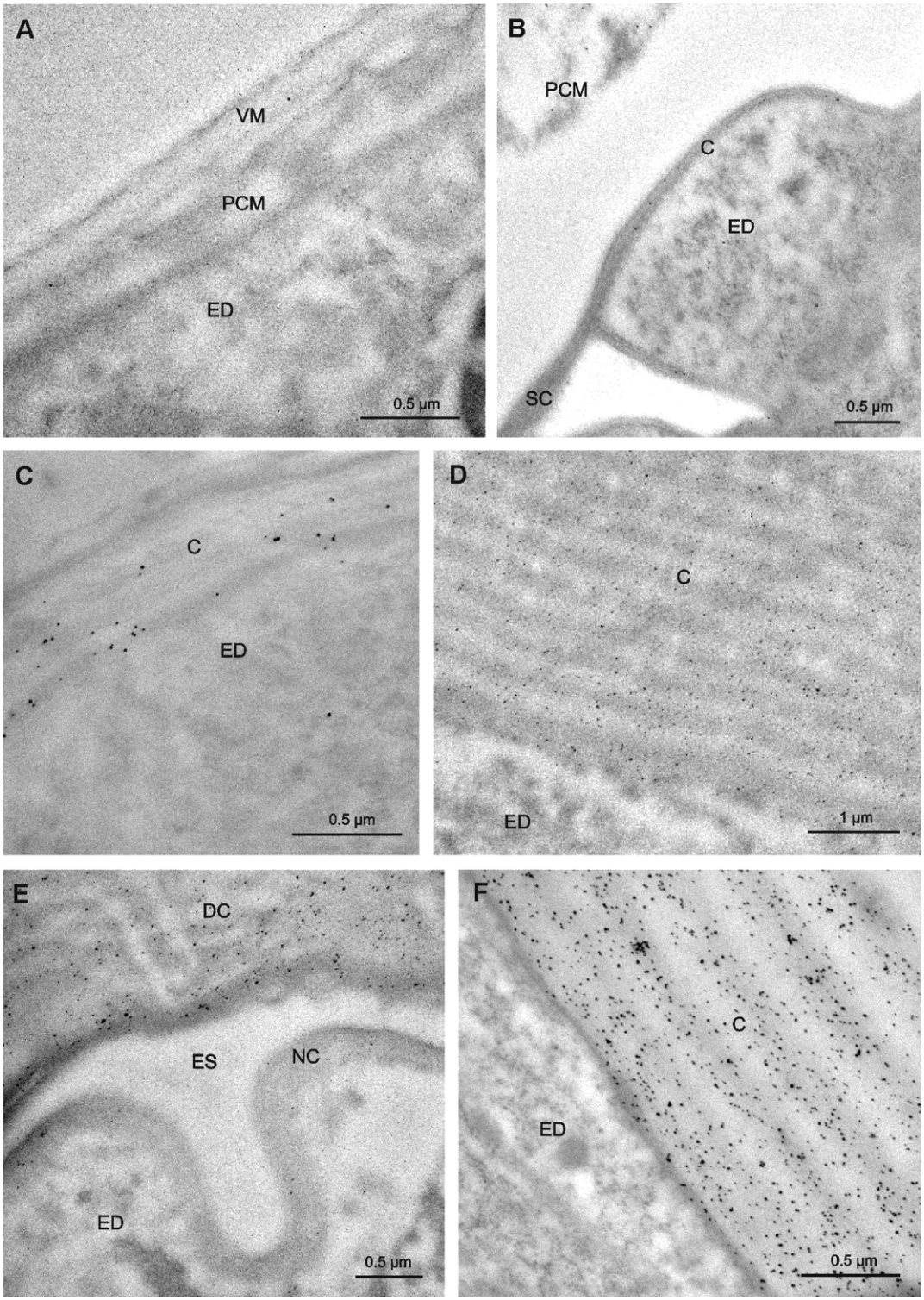

Fig. 7. Localization of *N*-acetyl-glucosamine oligomers, including chitin, by lectin WGA-gold labelling. A. Late embryo (stage 16). No labelling in the precuticular matrix. B. Late embryo (stage 18). A sparse WGA-gold labelling is observed in the pre-cuticular matrix. Moderate labelling is evident in the line of the newly forming cuticle. C. Newly hatched early marsupial manca. The exoskeletal cuticle is conspicuously labelled. D. Advanced early marsupial manca. Binding of WGA-gold is very intense in the exoskeletal cuticle. E. Late marsupial manca. WGA-gold labelling is observed in the detached cuticle and in the thin newly forming cuticle. F. Adult. Exoskeletal cuticle is intensely labelled. Cuticle (C), detached cuticle (DC), ecdysial space (ES), epidermal cell (ED), new cuticle (NC), precuticular matrix (PCM), epicuticular scale (SC), vitelline membrane (VM).

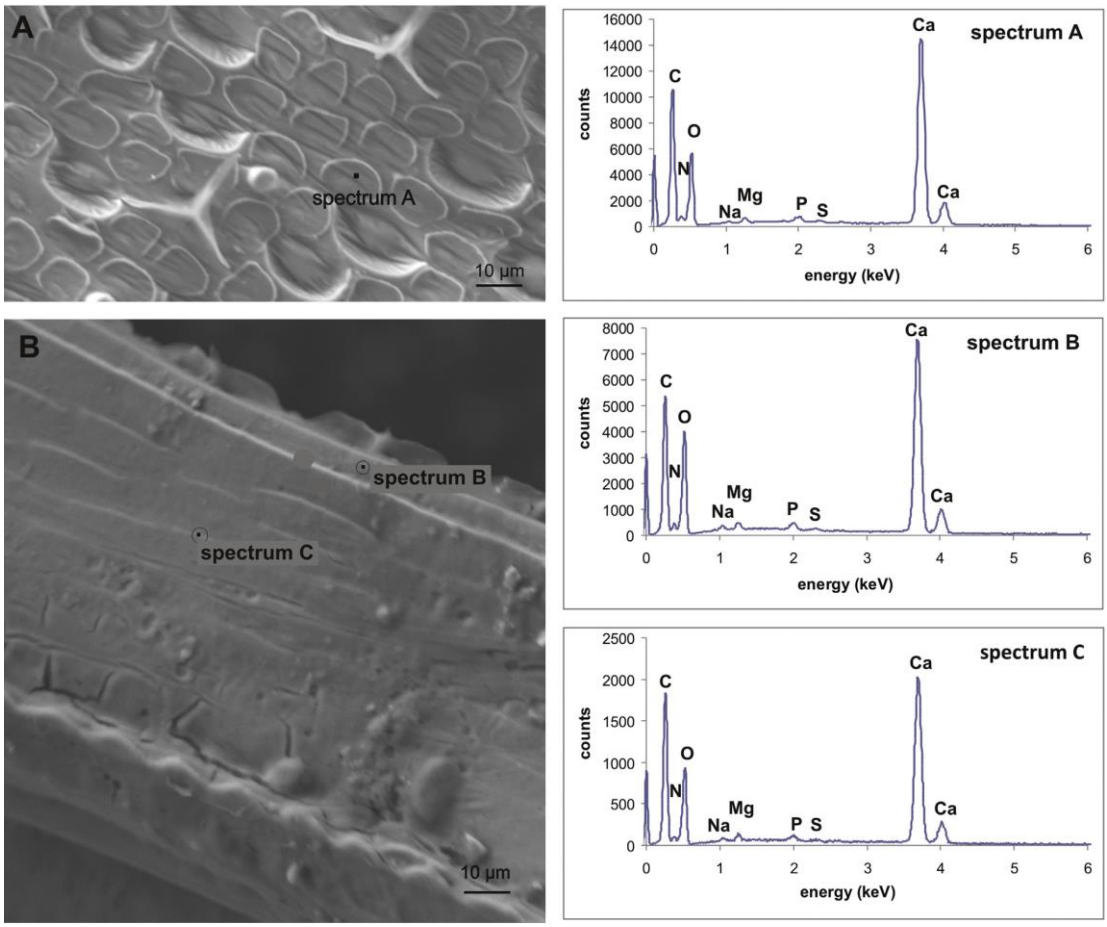

Fig. 8. Tergite cuticle of adult *P. scaber*. A. SEM micrograph of the cuticle surface, depicting the location where EDXS spectrum A was recorded. B. SEM micrograph of the transversely fractured cuticle and locations where EDXS spectra B and C were recorded. The obtained EDXS spectra A, B and C show prominent contents of calcium. Additional elements that were detected include phosphorus, sulphur, magnesium, carbon and oxygen. Ca peaks are several times higher than P peaks.

compositions of tergites were recorded in adult *P. scaber* by Hild et al. (2008) and in other isopod species, i.e. *Armadillidium vulgare* (Hild et al., 2008), *Titanethes albus* (Hild et al., 2009) and *Tylos europaeus* (Seidl et al., 2011). This observation is in agreement with the report of Ouyang and Wright (2005), which performed atomic absorption spectroscopy (AAS) measurements of whole embryo and manca calcium contents in isopod *A. vulgare* and reported a major and rapid increase in total calcium during middle stages of marsupial mancae development. They presumed that calcium uptake by manca is due to the ingestion of marsupial fluid observed in their study. Thus, calcium from marsupial fluid may also contribute to cuticle calcification in marsupial manca.

Our results are summarized in a graphic representation (Fig. 11), showing the presence and features of precuticular matrices and exoskeletal cuticles in the examined developmental stages. We use the term precuticular matrix as significant structural dissimilarities to the typical arthropod exoskeletal cuticle are evident. The term precuticular also implies that this matrix precedes cuticle formation. We use the term cuticle here for the matrices that display general characteristics of crustacean exoskeletal cuticle: a chitin-based scaffold, alignment to the cell membrane contour, generally uniform thickness, elaboration of cuticular scales, differentiation in epi- and procuticle and helicoidal, twisted plywood arrangement of chitin–protein fibres (Neville, 1984; Compere et al., 2004; Dillaman et al., 2013). In summary, several epidermal extracellular matrices are formed during intramarsupial development of isopod crustacean *P. scaber*. Renewal of these transitory matrices temporally corresponds to growth related morphological changes and possible osmoregulatory capacity changes of embryos and larvae. As these events proceed rapidly during development, formation and removal of the epidermal matrices occur in relatively short intervals. In the mid-stage embryo, at least two successive precuticular matrices are formed. Shedding of the precuticular matrices is observed during transition to the late-stage embryo and

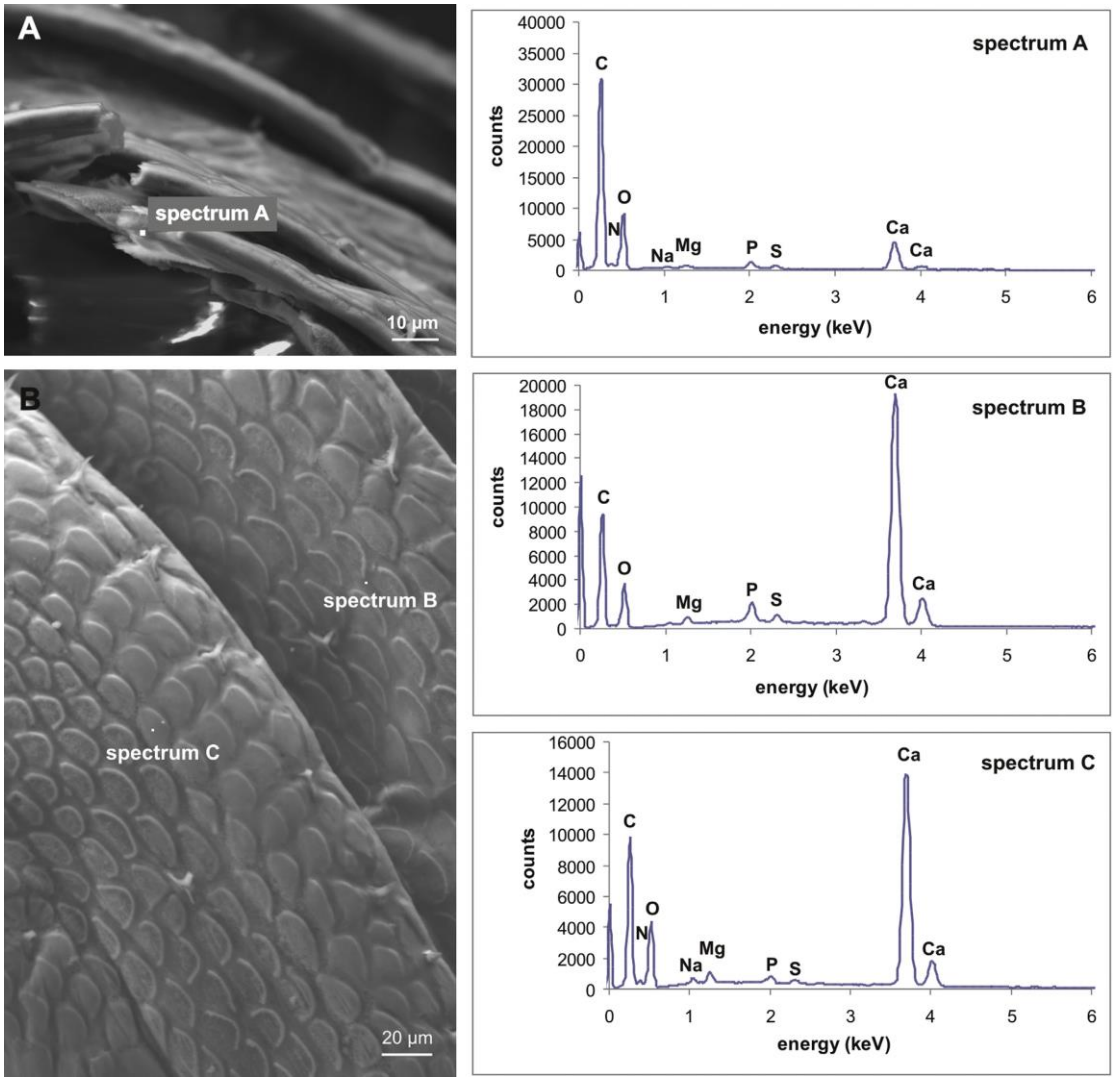

Fig. 9. Tergite cuticle of *P. scaber* advanced early-stage marsupial manca. A. EDXS spectrum A was obtained from the location depicted in the SEM micrograph of the transversely fractured cuticle. B. EDXS spectra B and C were obtained from the locations depicted in the SEM micrograph of the manca surface. All spectra show conspicuous calcium peaks and additional principal peaks of phosphorus, magnesium, sulphur, oxygen and carbon. Ca peaks are evidently higher than P peaks.

during hatching of the embryo. The chitin-based cuticle that displays structural characteristics of the crustacean exoskeletal cuticle is formed underneath the precuticular matrix in the last period of embryogenesis. The first ecdysis occurs approximately in the period of embryo hatching from the vitelline membrane. The manca secretes a new cuticle that displays differentiated structure of the epi- and the procuticle, a conspicuous labelling with the WGA-gold probes and calcium sequestration. In sequential marsupial manca development the cuticle architecture, thickness, labelling with the WGA lectin–gold complexes and EDXS analyses reveal that the exoskeleton structure and composition are already similar in this stage to those in adults. Before the release of the manca from the marsupium, the cuticle apolysis and disintegration are observed. In some specimens a new cuticle is formed, indicating exuviation of manca shortly after release from the marsupium. Our results show gradual formation and calcification of the exoskeleton during *P. scaber* larval development within the marsupium. The exoskeleton provides support for the muscles and is involved in the animal mobility which was observed within the marsupium (Mrak et al., 2012). Elaborated and calcified cuticle also protects the larvae against potential physiological stresses, including osmotic and ionic variations in the marsupial environment.

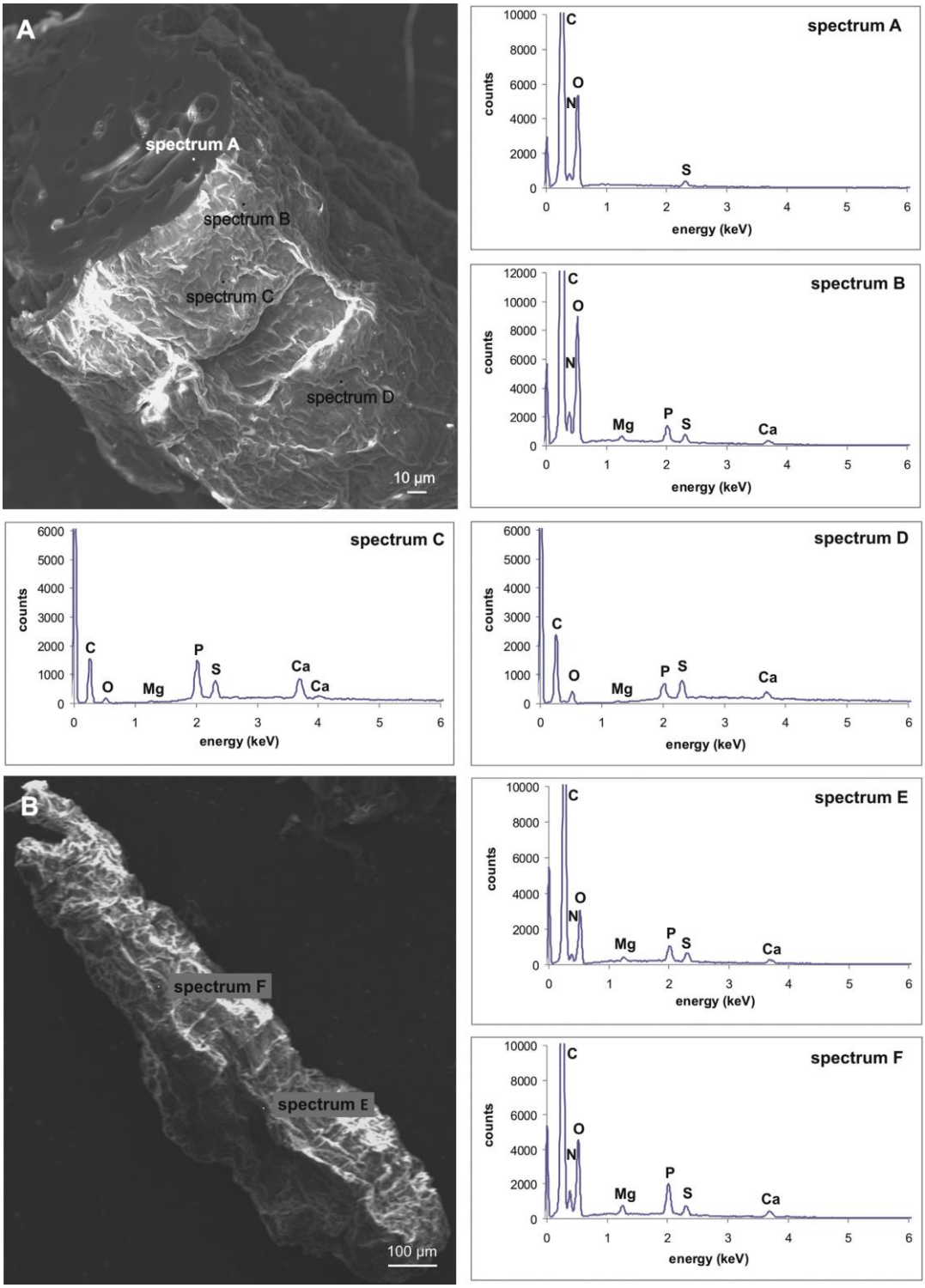

Fig. 10. Newly hatched early marsupial manca of *P. scaber*. A. SEM micrograph of the transversely fractured manca, depicting the locations where EDXS spectra A–D were obtained. B. SEM micrograph of the intact manca, depicting the locations in the dorsal side of the manca, where EDXS spectra E and F were obtained. The spectra of the manca surface (spectra B–F) show calcium and phosphorus contents, calcium peaks are always lower than phosphorus peaks. Additionally, magnesium, sulphur, carbon and oxygen were detected. Spectrum A was obtained from the internal tissue as a reference.

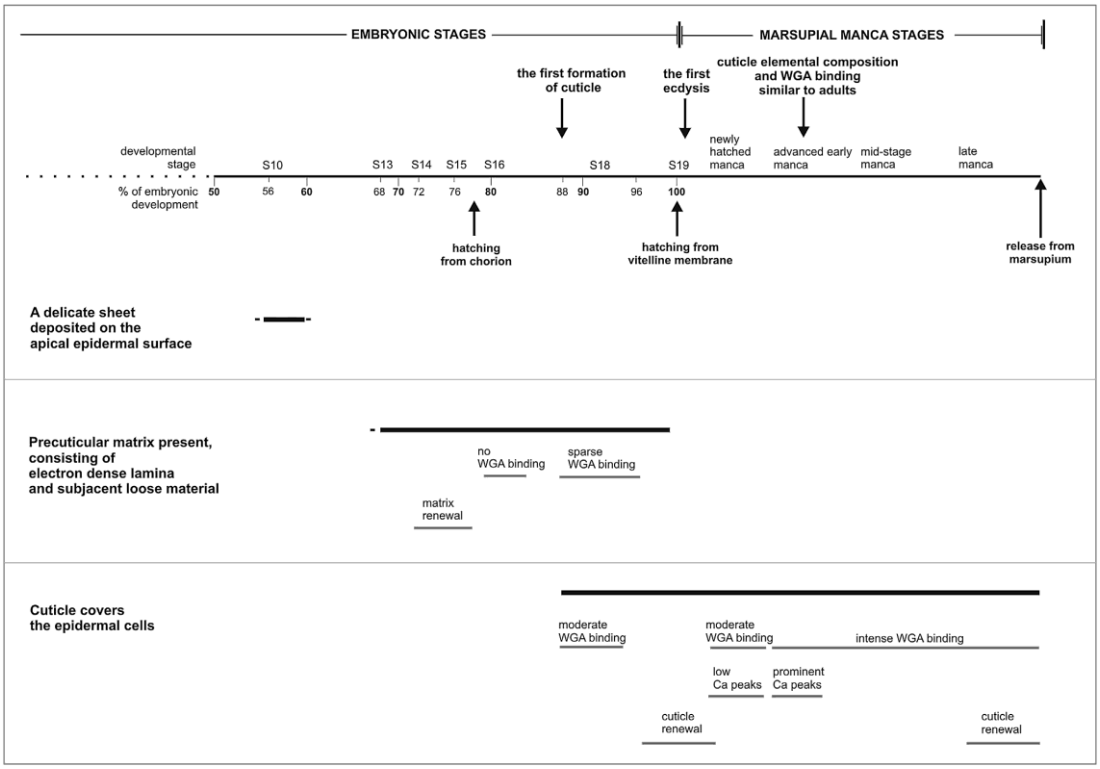

Fig. 11. Graphic representation of exoskeletal cuticle differentiation according to the embryonic and larval intramarsupial developmental stages of the isopod *Porcellio scaber*. Axis representing the successive developmental stages (S10–S19) and the percentage of development runs from left to right and selected significant events are indicated by arrows. The presence of the matrices in certain developmental stages is represented by the thick horizontal lines. Additional characteristics of the precuticular matrices and cuticles examined in this study are specified by descriptions below the thick lines.

Acknowledgement

This work was supported by the Slovenian Research Agency (ARRS), grant No. 1000-11-310087 and grant No. P1-0184. We thank Jožica Murko Bulić for laboratory assistance. We are very grateful to reviewers for carefully reading the manuscript and for their constructive suggestions.

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2.1.4 Povezave eksoskeleta s specializiranimi epitelnimi celicami (tenociti) in mišicami pri rakih enakonožcih v levitvi

Exoskeleton anchoring to tendon cells and muscles in molting isopod crustaceans

Nada Žnidaršič, Polona Mrak, Magda Tušek-Žnidarič in Jasna Štrus

Zookeys, 2012, 176: 39-53

Specializirane mehanske povezave med eksoskeletom in spodaj ležečimi mišicami pri členonožcih so kompleksne asociacije zunajceličnega matriksa, celičnih stikov in citoskeletnih elementov. Omogočajo močno mehansko pritrditev kompleksnega kutikularnega matriksa na apikalno plazmalemo specializirane epitelne celice (tenocita) in preko povezave tenocita z mišicami, prenos mišične napetosti na eksoskelet. Ultrastrukturna organizacija teh kompleksov v fazi levitve je pomembna tako z vidika ohranjanja integritete integumenta kot z vidika zmožnosti gibanja. Ultrastruktura povezav eksoskeleta in mišic pri embrijih in mankah rakov enakonožcev ni raziskana, zelo malo podatkov je tudi o odraslih živalih v levitvi.

V raziskavi smo opisali ultrastrukturo povezav med eksoskeletom in mišicami pri kopenskih rakih enakonožcih, in sicer pri poznem embriju pred izleganjem, marzupijski manki in odraslih v fazi predlevitve, ko epidermis pokrivata obe kutikuli, novo nastajajoča kutikula in stara odmaknjena kutikula. Pri odrasli mokrici vrste *Ligia italica* in pri omenjenih razvojnih stadijih vrste *Porcellio scaber* smo pokazali, da je na mestih mišičnih pritrditev stari eksoskelet obsežno povezan s spodaj ležečim epitelom preko številnih navpično razporejenih vlaken. Vlakna potekajo od tenocita skozi novo kutikulo in levitveni prostor ter vse do distalnih slojev stare kutikule. To kaže, da med menjavo eksoskeleta verjetno poteka reorganizacija vlaken in njihovih povezav s kutikulo, ne pa nastanek novih vlaken. Ta vlakna so verjetno zadnje povezave eksokutikule z epitelom pred levitvijo in skupaj s tenociti in mišičnimi celicami omogočajo gibanje živali v tej fazi. Strukturno ogrodje mišičnoskeletnih povezav je v analiziranih marzupijskih razvojnih stadijih v osnovi že podobno strukturi pritrditve mišice na eksoskelet pri odraslih členonožcih, kar kaže na njihovo vlogo pri gibanju živali znotraj valilnika. Tenociti so pri poznem embriju pred izleganjem in pri marzupijski manki strukturno diferencirani, morfološko podobni celicam odraslih, z apikalno-bazalno ureditvijo mikrotubulov, obsežnimi povezavami z mišicami in vlaknastimi povezavami z apikalno ležečo kutikulo nad njimi. Stik med tenocitom in mišično celico je pri obeh razvojnih stadijih oblikovan v vzorec cikcak in poteka vzdolž celotne površine bazalne membrane tenocita. Pri marzupijski manki je strukturno popolnoma izoblikovan. Pri embriju pred izleganjem je citoplazemski material pod bazalno membrano tenocita svetlejši in tanjši v primerjavi z stikom pri odraslih, iz česar predvidevamo, da stik še ni popolnoma diferenciran.

ZooKeys 176: 39–53 (2012)
doi: 10.3897/zookeys.176.2445
www.zookeys.org

RESEARCH ARTICLE

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Exoskeleton anchoring to tendon cells and muscles in molting isopod crustaceans

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Academic editor: *S. Sfenthourakis* | Received 25 November 2011 | Accepted 7 February 2012 | Published 20 March 2012

Citation: Žnidaršič N, Mrak P, Tušek-Žnidarič M, Štrus J (2012) Exoskeleton anchoring to tendon cells and muscles in molting isopod crustaceans. In: Štrus J, Taiti S, Sfenthourakis S (Eds) Advances in Terrestrial Isopod Biology. ZooKeys 176: 39–53. doi: 10.3897/zookeys.176.2445

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 arhitecture 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 integumental 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 premolt *Ligia italica* and in prehatching embryos and premolt 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

Introduction

The arthropod exoskeleton performs diverse functions, including mechanical support, sensing, prevention of desiccation and protection against pathogens and predators. Locomotion of these animals is based on extensive connections between exoskeleton and muscular system. The exoskeleton consists of a complex chitin-protein matrix, secreted by a single-layered epithelium. The chitin-protein matrix is either non-calcified or calcified, as in insects and crustaceans, respectively. Specialized epithelial cells, named tendon cells, are the sites of firm mechanical connections between exoskeleton and underlying tissues (Noble-Nesbitt 1963, Mellon 1992, Lai-Fook and Beaton 1998, Bitsch and Bitsch 2002). Apical membranes of tendon cells are anchored to the matrix of the cuticle and their basal surfaces are attached to muscle cells underneath (Fig. 1). A prominent ultrastructural characteristic of tendon cells are extensive bundles of microtubules, that stretch between the apical cell membrane facing the cuticle and the basal membrane engaged in the myotendinous junction. Thus, the muscle is mechanically linked to epidermis and cuticle by a complex network of cytoskeletal and junctional elements. In addition to their essential role in animal locomotion, muscle cells are expected to play important role at molting, as force-generators for movements that facilitate the exuviation.

The ultrastructure, molecular composition and differentiation of specialized anchoring complexes between exoskeleton, tendon cells and muscle cells were extensively studied in an insect *Drosophila melanogaster*, with emphasis on myotendinous junction characterization (Volk 1999, Brown 2000, Volk 2006, Schweitzer et al. 2010). Apart from *Drosophila*, there is limited data available on the fine structure and constitutive elements of exoskeleton - muscle attachment sites in other arthropods. Studies on the ultrastructural organization of connections between exoskeleton and muscles in the phases of new cuticle formation were performed in some insect species (Lai-Fook 1967, Caveney 1969, Lai-Fook and Beaton 1998) and in two groups of crustaceans: in *Euphausia superba* (Crustacea: Euphausiacea) by Buchholz and Buchholz (1989) and in podocopid ostracods by Yamada and Keyser (2009). Premolt is the unique stage preceding exuviation in which the integumental epithelium is covered by both, the newly forming cuticle apposed to the epithelial cells and the old detached cuticle above the ecdysal space. The key features of this stage in crustaceans are simultaneous disintegration of the old cuticular matrix and new cuticle formation, including secretion and recycling of organic constituents, massive calcium fluxes and modification of epithelial cells shape, size and ultrastructure (Roer and Dillaman 1993, Ziegler 1997, Compere et al. 1998, Štrus and Blejec 2001, Luquet and Marin 2004, Dillaman et al. 2005, Ziegler et al. 2005, Žnidaršič et al. 2010). Exoskeleton renewal inevitably involves the establishment of connections between the new cuticle and muscles. To supplement the knowledge on the ultrastructure of these connections and to address the issues of general principles vs. specializations of their architecture and reorganization related to molting, several species from different environments need to be studied in this respect. The exoskeleton renewal takes place during development and molting in adult specimens. There are no detailed ultrastructural data on exoskeleton anchoring to tendon cells and muscles in molting adults and in

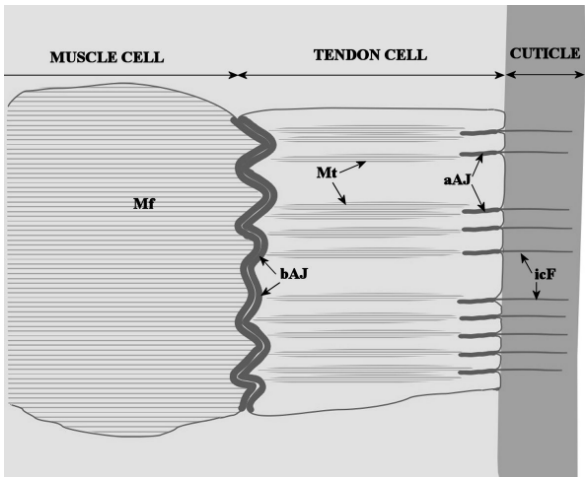

Figure 1. A scheme showing the general architecture of the muscle attachment to the epidermis in arthropods (adapted from Mellon 1992, Lai-Fook and Beaton 1998, Bitsch and Bitsch 2002 and Subramanian et al. 2003). Specialized epithelial cells, named tendon cells, are anchored apically to the cuticle and basally to the muscle cell. **aAJ** apical adherens junction **bAJ** basal adherens junction **icF** intracuticular fibers **Mf** myofilaments **Mt** microtubules.

marsupial stages of isopod crustaceans. The embryonic development of terrestrial isopod crustaceans and hatching of embryos to marsupial mancas take place inside the female brood pouch (marsupium) and were decribed in *Porcellio scaber* from the view of overall morphology and digestive system morphogenesis, while the issue of cuticle anchoring was not addressed (Štrus et al. 2008, Wolff 2009, Milatovič et al. 2010). Fertilized eggs are released into the brood pouch, where the entire embryonic development takes place. The final phase in the embryonic development is hatching. Newly hatched animals are termed marsupial mancas and they stay inside the brood pouch for up to ten days as described in *P. scaber* females reared in the laboratory (Milatovič et al. 2010).

Here we report new data on the ultrastructural architecture of anchoring complexes comprising exoskeleton, tendon cells and muscles in adult premolt isopod crustaceans, in premolt marsupial mancas and in prehatching embryos. Our study is focused primarily to connections between the complex matrix of the exoskeleton and tendon cells, modified epithelial cells at the sites of muscles attachment. To the best of our knowledge, the exoskeleton anchoring to underlying tissues in embryos and marsupial mancas of crustaceans has not been characterized before and its ultrastructural organization is presented here. Comparative evaluation of the results with respect to the other arthropods, particularly to insect model organism *D. melanogaster,* is presented. The involvement of these anchoring connections in molting and in intramarsupial motility is discussed.

Methods

Specimens of *Ligia italica* Fabricius, 1798 (Crustacea: Isopoda) were collected at the Piran Bay coast in Slovenia. Animals were inspected for ventral sternal deposits and

premolt adult specimens were anaesthetized. The dorsal parts of pereonites were isolated, fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) and postfixed by 1% OsO_4. After washing and dehydration in a graded series of ethanol, samples were embedded in an epoxy resin mixture. Semithin sections were stained with Azure II. – Methylene blue. Ultrathin sections were either imaged non-contrasted or contrasted with uranyl acetate and lead citrate.

Specimens of *Porcellio scaber* Latreille, 1804 (Crustacea: Isopoda) prehatching embryos and marsupial mancas were isolated from brood pouches of females maintained in laboratory culture. Determination of intramarsupial developmental stages was performed as described in Milatovič et al. (2010). Isolated embryos and mancas were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). Prior to fixation, the vitelline membrane of embryos was carefully perforated with a thin needle. After washing with 0.1 M cacodylate buffer, the samples were postfixed in 1% OsO_4 for 2 hours, washed again and dehydrated in a graded series of ethanol. Specimens were embedded in Agar 100 resin. Prior to embedding, mancas were perforated with a thin needle for better infiltration of resin. Semithin sections were stained with Azure II. - Methylene blue. Ultrathin sections were contrasted with uranyl acetate and lead citrate.

Light microscopy was performed by AxioImager Z.1 microscope (Zeiss) equiped with an AxioCam HRc camera and Axiovision software. Ultrastructural imaging was performed by CM 100 transmission electron microscope (Phillips) equiped with a BioScan 792 digital camera (Gatan) and Digital Micrograph software.

Results

Adult premolt specimens

The ultrastructural architecture of the exoskeleton – muscles attachment regions was analysed in the dorsal parts of pereonites in adult premolt *Ligia italica*. The pre-ecdysial cuticle was tightly connected to the underlying tendon cells and muscles already in the early premolt (Fig. 2). Extensive connections were established between the chitin-protein matrix of the newly forming cuticle and the apical parts of the tendon cells in the early phase of cuticle elaboration. These matrix – cell linkages consisted of numerous fibrous structures. The most intriguing result was that the fibers traversed the entire new cuticle, spanned the whole ecdysal space and protruded deep into the old cuticle (Figs 2B, C). They extended from the tendon cell apex up to the exocuticular layer in the old cuticle. The continuity of these fibers was clearly followed in some sections, revealing a direct mechanical connection of the detached exoskeleton to epithelium in these regions. Fibrous structures were arranged in parallel to one another and in the same direction as microtubules arrays in the tendon cells and myofilaments arrays in the muscle cells. This direction is roughly perpendicular to the body surface. Fibrous connections followed approximately straight lines and did not display any branching or prominent curvatures. General architecture of the preecdysal cuticle at muscle

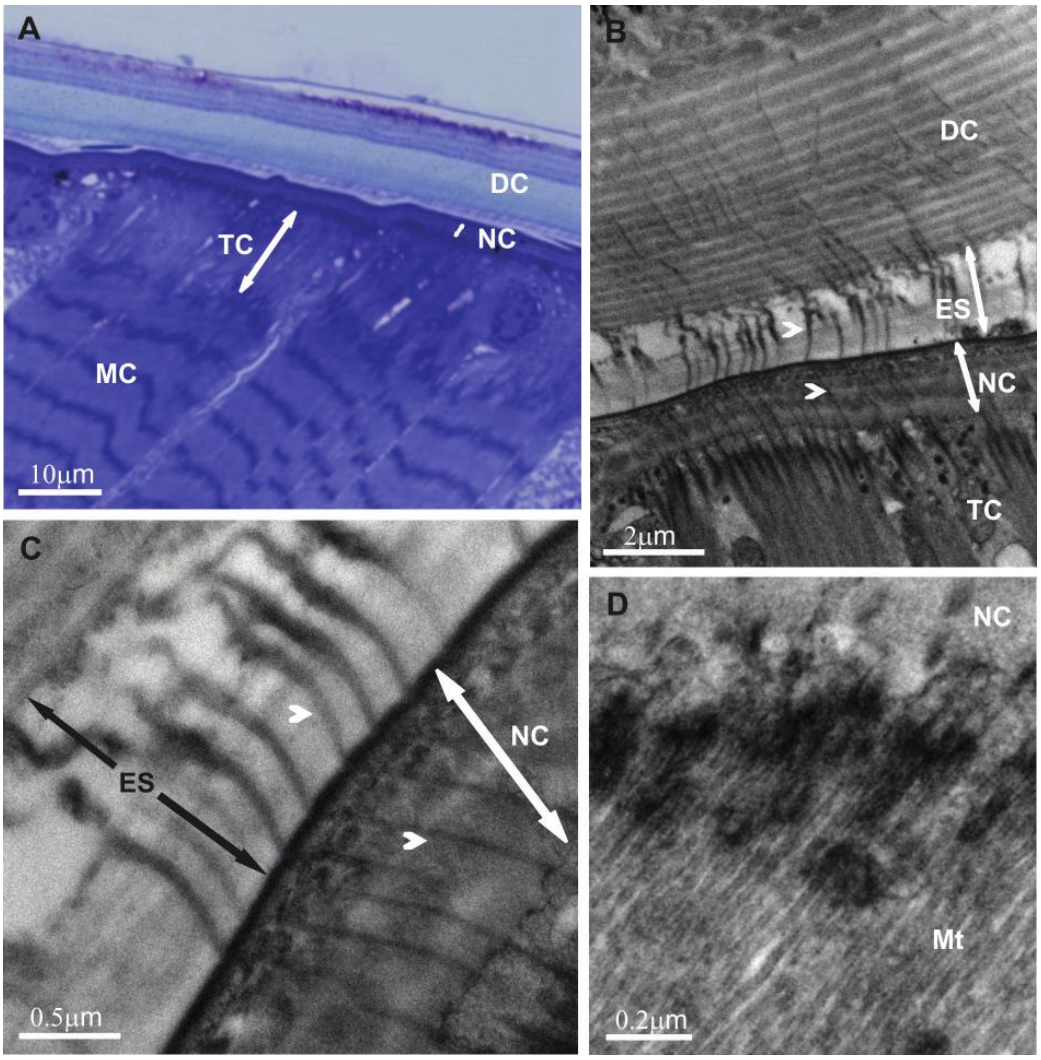

Figure 2. Exoskeleton – muscle attachment in the dorsal parts of pereonites in adult premolt *Ligia italica*. Overview of the muscle attachment in a specimen with a detached and a newly forming cuticle (**2A** a semithin section). The new cuticle is extensively connected to the underlying tendon cells already in the early premolt. Fibrous connections running from the apical region of tendon cells through the new pre-ecdysal cuticle and ecdysal space up to the exocuticle of the detached exoskeleton are evident **(2B, C)**. Parallel arrays of microtubules and apical electron dense plaques are characteristic for tendon cells **(2D)**. **DC** detached cuticle **NC** new cuticle **ES** ecdysal space **MC** muscle cell **TC** tendon cell **Mt** microtubules; arrowheads – fibrous connections.

attachment sites was similar to that in the other regions, displaying epicuticular and exocuticular layers and characteristic pattern of chitin-protein fibers arrangement.

Extensive parallel arrays of microtubules inside the tendon cells were aligned in the apical to basal direction (Fig. 2D). A concourse of microtubules towards the cytoplasmic densities at the apical membrane (hemidesmosome-like structures) of tendon cells was evident. On the basal side the microtubules were positioned close to the electron dense plaques along the basal membrane, engaged in myotendinous junction.

Prominent anchoring junctions were evident between muscle cells and tendon cells (Fig. 3). The entire basal membrane of the tendon cell was intensely folded in a zigzag pattern, exactly matching the folding of the muscle cell sarcolemma beneath. Both cell surfaces contributing to this junction were closely apposed and the intervening layer of the extracellular matrix material was not conspicous. The complex connection between these two cells in premolt specimens corresponds to characteristic design in animals with one complete cuticle (Supplementary figure), comprising electron dense plaques beneath both cell membranes and extracellular material in the narrow intercellular space.

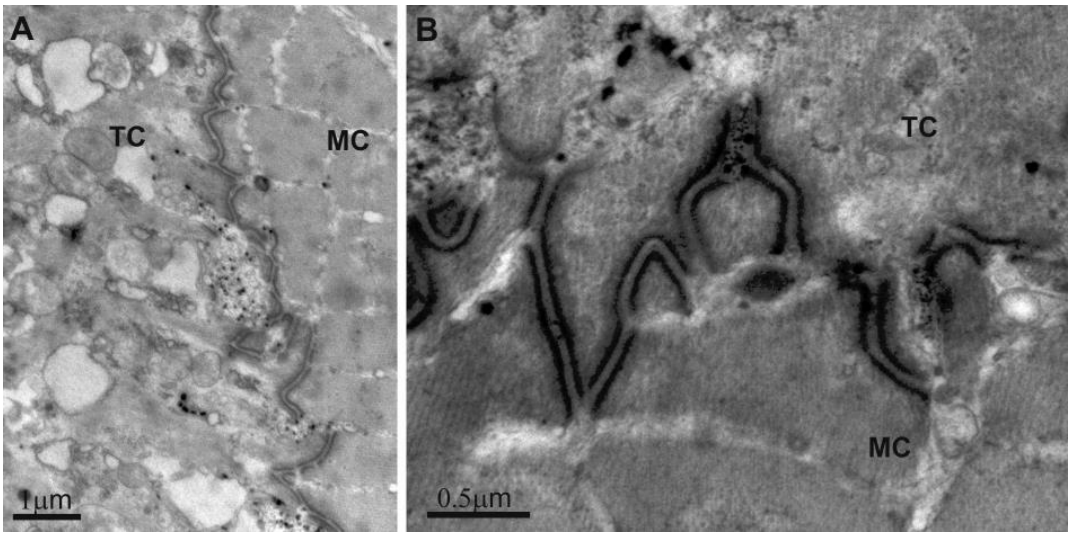

Figure 3. Myotendinous junction in the dorsal parts of pereonites of premolt adult *Ligia italica* is an extensive anchoring junction. The entire zigzag folded basal and apical membranes of tendon and muscle cells, respectively, are engaged in this outstanding intercellular mechanical connection **(3A)**. Prominent electron dense cytoplasmic plaques are evident along both cell membranes, separated by a thin layer of extracellular matrix **(3B)**. **MC** muscle cell **TC** tendon cell.

Intramarsupial premolt animals

Development of *Porcellio scaber* embryos and marsupial mancas involves renewal of the exoskeleton. Prehatching embryo and several marsupial mancas displaying morphological attributes of premolt were analysed in this study. The detachment of the old cuticle and disintegration of the basal parts of the detached cuticle were identified in the integument of these specimens. In addition, the substantial new cuticle formation was evident in marsupial mancas. The exoskeleton in the prehatching embryo and in the premolt marsupial mancas was extensively connected to tendon cells and muscles underneath and extensive intercellular junctions between muscle cells and tendon cells were established (Fig. 4).

Numerous fibrous connections between the detached cuticle and the apical membrane of tendon cells were evident in all premolt intramarsupial animals examined (Fig. 5). The newly forming cuticle in marsupial mancas, consisting of epicuticle and a few layers of the pre-ecdysal procuticle, was mechanically connected to tendon cells.

Tendon cells ultrastructurally resembled the adult arthropod tendon cells and were characterized by apicobasal arrays of microtubules. Apically, the microtubules were found close to oblong electron dense regions (hemidesmosome-like structures), aligned in the same direction (Figs 5D–F and Fig. 6). In longitudinal and in oblique sections the profiles of microtubules were evidenced in close proximity to the electron dense plaques of junctions along the tendon cell basal membrane in both, prehatching embryos (Figs 7A, B) and marsupial mancas (Figs 7C, D).

Myotendinous junctions displayed a characteristic zigzag outline, occupying the entire tendon – muscle interface (Fig. 7). From the structural point of view, the myotendinous junctions in marsupial mancas closely resembled these junctions in adult

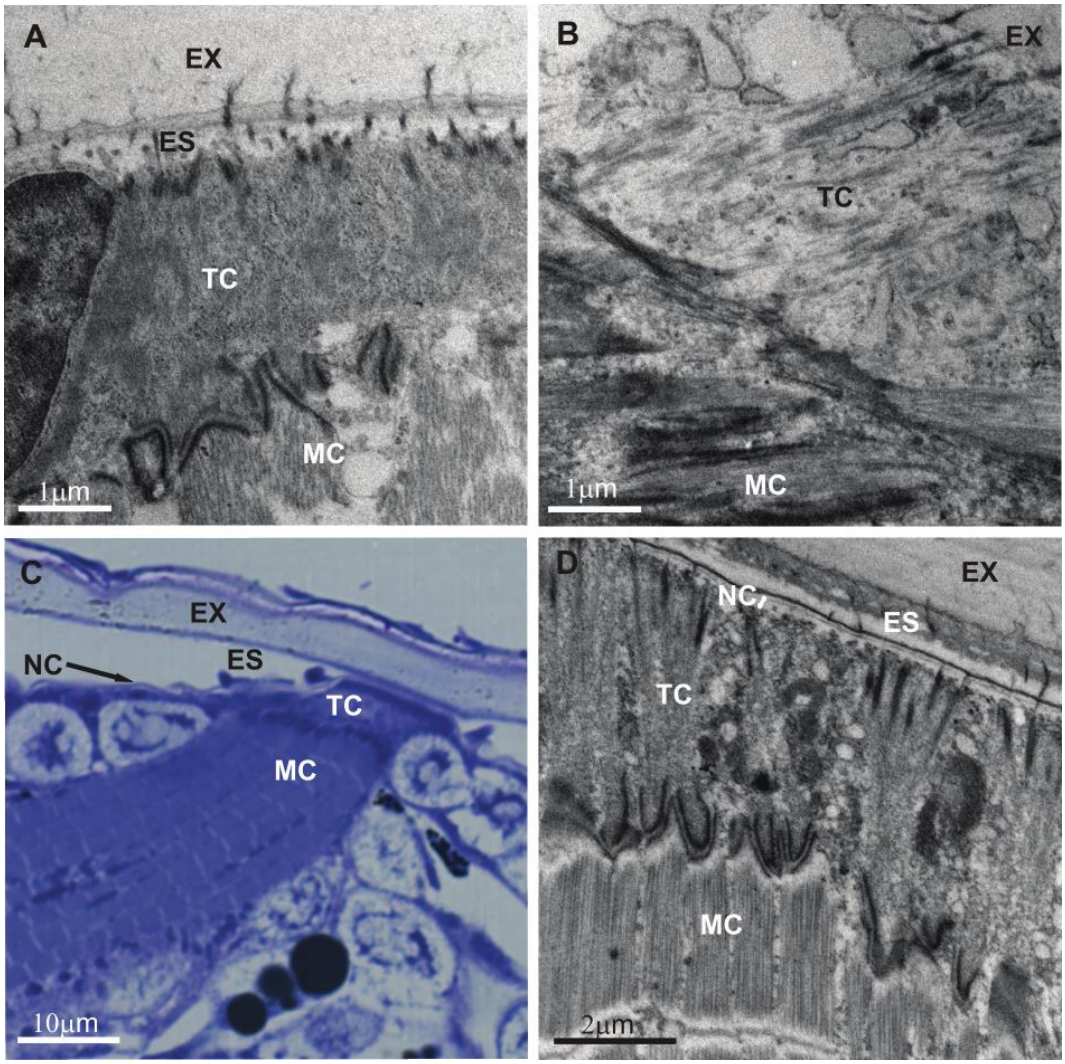

Figure 4. Overview of exoskeleton anchoring to tendon cells and muscles in intramarsupial developmental stages of *Porcellio scaber*. In prehatching embryos **(4A, B)** and in premolt marsupial mancas (**4C** a semithin section and **4D**) the connections between the exoskeleton, tendon cells and muscle cells were already established. **EX** exoskeleton **ES** ecdysal space **NC** new cuticle **TC** tendon cell **MC** muscle cell.

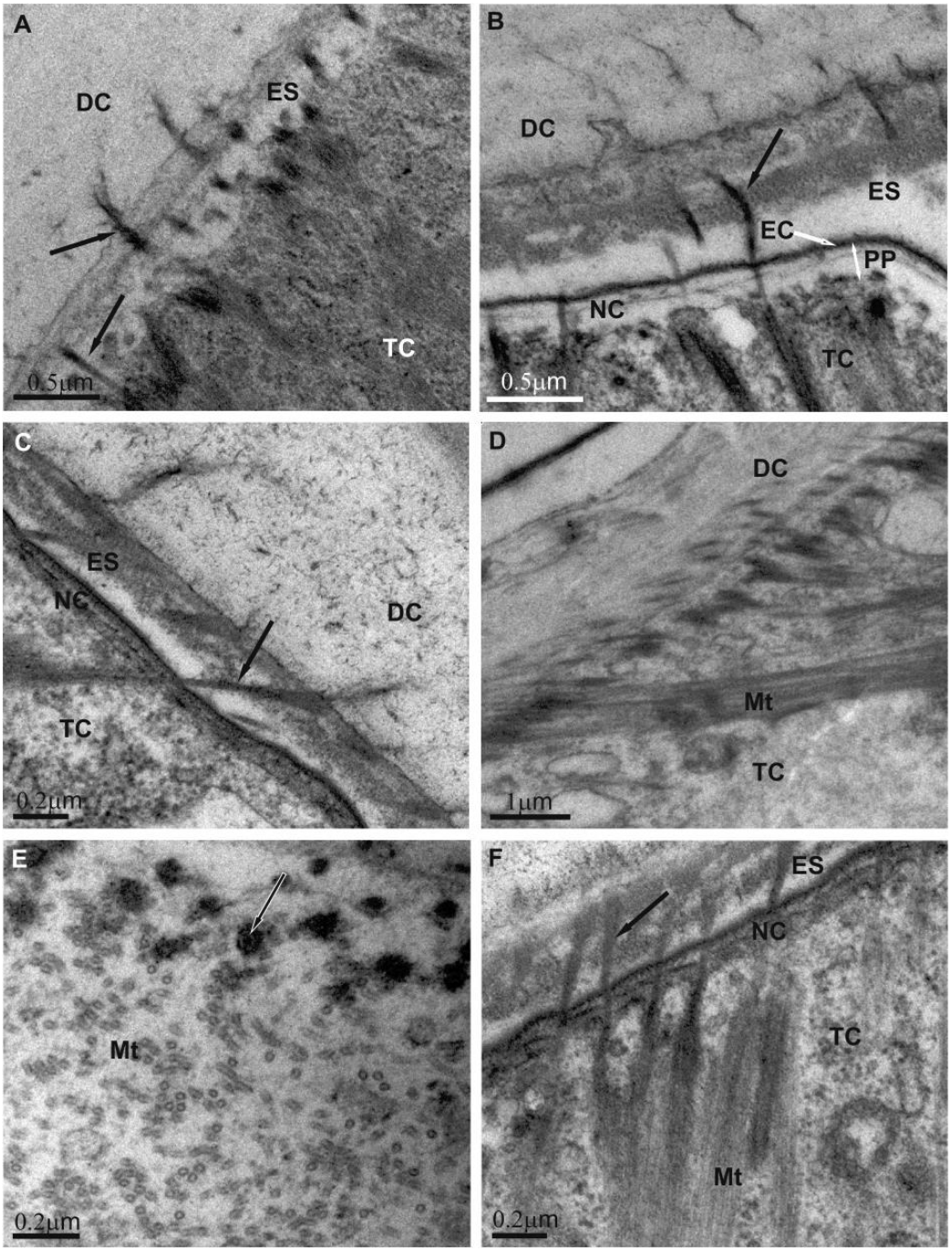

Figure 5. Anchoring junctions between the detached cuticle and the apical part of tendon cells in the prehatching embryo **(5A, D, E)** and marsupial mancas **(5B, C, F)** of *Porcellio scaber* (ultrathin cross-sections). The newly forming cuticle in marsupial mancas, consisting of the epicuticle and pre-ecdysal procuticle, was mechanically connected to tendon cells. Numerous bundles of fibers (arrows) running from tendon cells through the new cuticle and ecdysal space into the detached cuticle are evident. Microtubules were found in close proximity to electron dense plaques at the apical surface of tendon cells. **DC** detached cuticle **NC** new cuticle **EC** epicuticle **ES** ecdysal space **PP** pre-ecdysal procuticle **TC** tendon cell **Mt** microtubules.

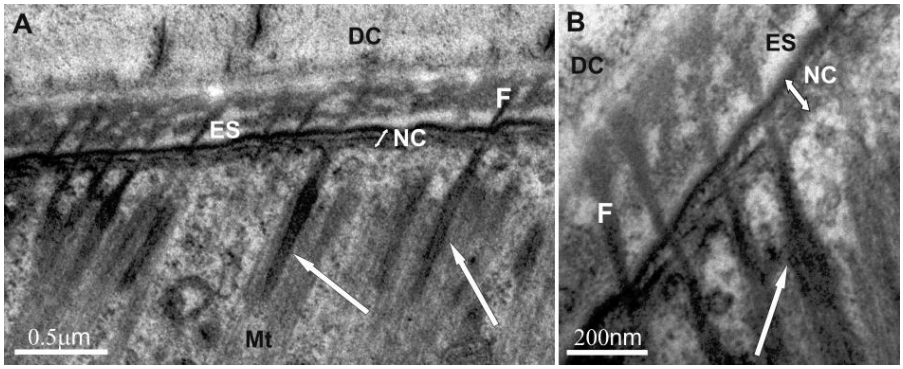

Figure 6. Electron dense plaques (hemidesmosome-like structures) at the apical surface of the tendon cells in the marsupial manca of *Porcellio scaber*. Electron dense plaques (arrows) are associated with microtubules **(Mt)** in the cytoplasm of the tendon cell and with the bundles of fibers **(F)** running through the ecdysal space **(ES)** on the opposite side. **DC** detached cuticle **NC** new cuticle.

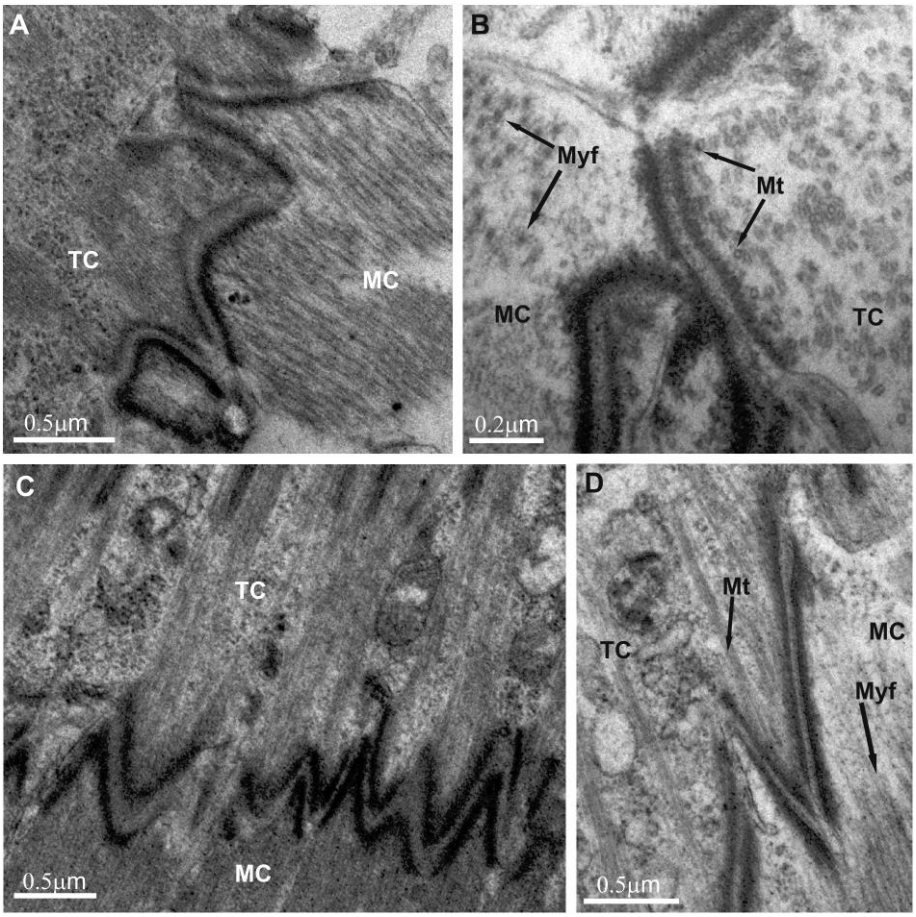

Figure 7. Myotendinous junction in the *Porcellio scaber* prehatching embryo **(7A, B)** and marsupial mancas **(7C, D)**. The cytoplasmic plaque of the anchoring junction at the basal membrane of tendon cell is more lucent and thinner than the accompanying plaque at the apical membrane of the muscle cell in the prehatching embryo **(7A, B)**. Microtubules of the tendon cells are in close proximity to the basal dense plaques. **TC** tendon cell **MC** muscle cell **Mt** microtubules **Myf** myofilaments.

arthropods, comprising a thin layer of extracellular material between cell membranes and dense cytoplasmic plaques of approximately equal densities and thicknesses below the both cell membranes (Figs 7C, D). On the other hand, in the prehatching embryo, the intracellular membrane-associated layer of dense cytoplasmic material contributing to the anchoring junction in a tendon cell was more lucent and thinner than that contributing to the junction in a muscle cell (Figs 7A, B).

Discussion

Specialized anchoring complexes between exoskeleton, tendon cells and force-generating muscle cells are essential features of the musculoskeletal system in arthropods (Noble-Nesbitt 1963, Mellon 1992, Lai-Fook and Beaton 1998, Bitsch and Bitsch 2002). A complex network of cytoskeletal and junctional elements constitutes the main scaffold of specialized mechanical connections between the calcified exoskeleton and underlying tissues in crustaceans (Mellon 1992). These elaborate connections between different types of cells and hierarchically structured extracellular matrix constitute a common functional network, enabling animal movements, and are thus involved also in animal responses to various physiological and environmental cues.

Our results show that the old exoskeleton is still mechanically attached to the underlying epithelium in the regions of muscle attachment sites for a certain period of the premolt phase in isopod crustaceans. We have observed massive arrays of fibers running from tendon cells through the new cuticle and ecdysal space up to the distal layers of the detached cuticle in adult premolt *L. italica* and in premolt intramarsupial specimens of *P. scaber*. The continuity of these fibers was clearly evidenced in both species. The fibers extending from the tendon cells deep into the cuticular matrix in non-molting specimens are known from previous studies of arthropods (Noble-Nesbitt 1963, Mellon 1992, Lai-Fook and Beaton 1998) and were also reported in some studies of molting insect species (Lai-Fook 1967, Caveney 1969, Lai-Fook and Beaton 1998) and in two studies of molting crustaceans (Buchholz and Buchholz 1989, Yamada and Keyser 2009).Different authors use different names to designate these fibers, which is rather confusing. They are termed muscle attachment fibers (Caveney 1969), tonofibrillae (Buchholz and Buchholz 1989, Lai-Fook and Beaton 1998, Tucker et al. 2004), tonofilaments (Subramanian et al. 2003), intracuticular fibers (Mellon 1992) and intracuticular rods (Criel et al. 2005). To the best of our knowledge, the macromolecules constituting these fibers have not been identified yet, neither in insects, nor in crustaceans. They are described as dense strands of cuticular material that project into the apical invaginations of tendon cells (Tucker et al. 2004). In contrast to comprehensively resolved molecular architecture of the tendon – muscle junction in *Drosophila* (Volk 2006, Schweitzer et al. 2010), the proteins that connect the apical surface of the epidermis to the cuticle are not known (Brown 2000). The ultrastructural organization of the fibrous connections between the detached cuticle and tendon cells described in our study for two isopod species closely resembles that reported for the

premolt *Euphausia superba* by Buchholz and Buchholz (1989) and for the premolt podocopid ostracods by Yamada and Keyser (2009). As in *E. superba* and in ostracods the new preecdysal cuticle in isopods is secreted while fibers maintain the connection between the old cuticle and epidermis. Yamada and Keyser (2009) in addition described the formation of the new cuticle in detail. They reported that the new epicuticle was deposited around the extended intracuticular fibers and suggested that the intracuticular fibers increase their length before the deposition of the new epicuticular material. The continuity of the fibers stretching from the tendon cells through the new cuticle and ecdysal space to the old cuticle is clearly evident in our images of premolt isopod integument. This indicates that the structural reorganization of the existing fibers occurs during premolt rather than extensive formation of the new fibers. This is also in agreement with previous reports on the continuity of the tonobrillae in molting insects (Lai-Fook 1967) and crustaceans (Buchholz and Buchholz 1989, Yamada and Keyser 2009). We consider that cuticle-muscle attachment complexes supposedly helping or enabling movements during shedding of the old cuticle are also involved in maintenance of integument integrity of the 'two-cuticle' stage in the premolt isopods. Fibers connecting the detached cuticle to the underlying tissues likely serve as final anchoring sites before exuviation. These anchoring connections to the underlying epidermis and muscles may also be involved in limited locomotory activity of the animal during molting. As the newly forming cuticle underneath is already extensively connected by such fibers to the apical membrane of tendon cells and indirectly to muscles below, at least elementary locomotion may thus be enabled also immediately after exuviation.

Apicobasally oriented microtubule arrays are formed in several types of polarized epithelial cells. Tucker et al. (2004) report that most microtubules in *Drosophila* tendon cells exhibit unusually large diameters of up to 30 nm, while in our study the usual microtubules of 24 nm in diameter were observed in the tendon cells of adult and intramarsupial isopods. Here we show that tendon cells in prehatching embryos and marsupial mancas of *P. scaber* already include a substantial apicobasally oriented transcellular arrays of microtubules, evidently engaged in myotendinous junctions and in apical anchoring of the cuticular matrix. Thus we consider that in the prehatching embryos and marsupial mancas the structural framework of musculoskeletal linkage is basically established and could assist in animal motions within the marsupium. This consideration is further supported by our observation that marsupial mancas display pronouced body movements inside the marsupial fluid and is supported also by the study of Milatovič et al. (2010), who reported that embryo hatching from the vitelline membrane involves swelling and active movement.

The myotendinous junction in the dorsal parts of pereonites in molting *L. italica* is a zigzag patterned junction of the tendon cell basal membrane and muscle cell sarcolemma, with an inconspicious layer of extracellular matrix inbetween. The entire basal surface and apical surface of tendon and muscle cell, respectively, contribute to this heterotypic adherens junction. Both interacting cell membranes are extensively folded, which increases the surface area of contact and contributes to enhanced mechanical resistance. The myotendinous connection in molting *L. italica* structurally resembles

that described in non-molting specimens and in *Drosophila*. The myotendinous junction in *Drosophila* is considered to be composed of two sets of hemiadherens junctions with an intervening layer of extracellular matrix material that has a substantial thickness in certain situations (Prokop et al. 1998, Tucker et al., 2004). In recent years the molecular machinery involved in the formation of these junctions has been increasingly elucidated. The *Drosophila* myotendinous connection is integrin dependant and involves transmembrane integrins that connect to protein ligands in the extracellular matrix and to the cytoskeleton inside the cell (Prokop et al. 1998, Delon and Brown 2008). This strategy is implemented in both, direct and indirect muscle attachments in *Drosophila*. The molecular composition of tendon cell – muscle cell junction has not been resolved in crustaceans, but similar principles involving integrins and associated linking proteins are expected to be implied.

The ultrastructural arhitecture of myotendinous junctions in the prehatching embryos and marsupial mancas of *P. scaber* analysed in this study is similar to the general structural outline of adult arthropod muscle attachments. In marsupial mancas it appears to be structurally fully elaborated, while in the prehatching embryo it may not be completely formed. The cytoplasmic plaques engaged in anchoring junctions at the basal membrane of the tendon cell in the prehatching embryo are markedly electron lucent and thinner as compared to the opposing cytoplasmic plaques in the muscle cells, while in adult arthropods the myotendinous anchoring junctions comprise cytoplasmic plaques of similar thicknesses and densities in both cells. A similar situation was observed in *Drosophila* in vitro culture of primary embryonic cells by Tucker et al. (2004), who reported that differentiating tendon cells are characterized also by thinner hemiadherens junction as compared to the associated muscle cell. Thus we consider that this structural feature may indicate the not completely differentiated junction. The functional significance of this is not yet clear.

Conclusions

Cell to cell and cell to matrix anchoring junctions, together with their associated cytoskeletal elements, are engaged in providing tissue structural scaffold and integrity, but more than that, they are increasingly discussed from the perspective of tissue and cell dynamics (Zaidel-Bar et al. 2010). These junctions undergo dynamic changes during development and during regeneration in adulthood. The overall general architecture of the exoskeleton-muscle attachment in isopod crustaceans described in our study is similar to that reported for other arthropods. We show here that elaborate anchoring junctions between the tendon cell apical membrane and the extracellular matrix provide attachment of the exoskeleton to the underlying tissues also during cuticle replacement in adult and in intramarsupial developmental stages of isopod crustaceans. Thus they contribute to integument integrity during molting and together with associ-

ated microtubules in tendon cells and myotendinous connections likely enable at least basic movements in this period. As cuticle replacement involves old cuticle detachment followed by the new chitin – protein matrix secretion at the apical membranes of epidermal cells, the rearrangement and remodeling of anchoring junctions between the tendon cells and cuticle are expected. The continuity of the fibers ranging from the tendon cells through the new cuticle and ecdysal space to the old cuticle was evident in premolt adult and intramarsupial isopods. This indicates that during premolt the reorganization of fibers and their associations with the cuticle takes place, rather then extensive formation of the new fibers. Our considerations are in agreement with previous reports on the continuity of tonofibrillae, which maintain the connection between the tendon cells and the old cuticle in molting insects (Lai-Fook 1967) and crustaceans (Buchholz and Buchholz 1989, Yamada and Keyser 2009).

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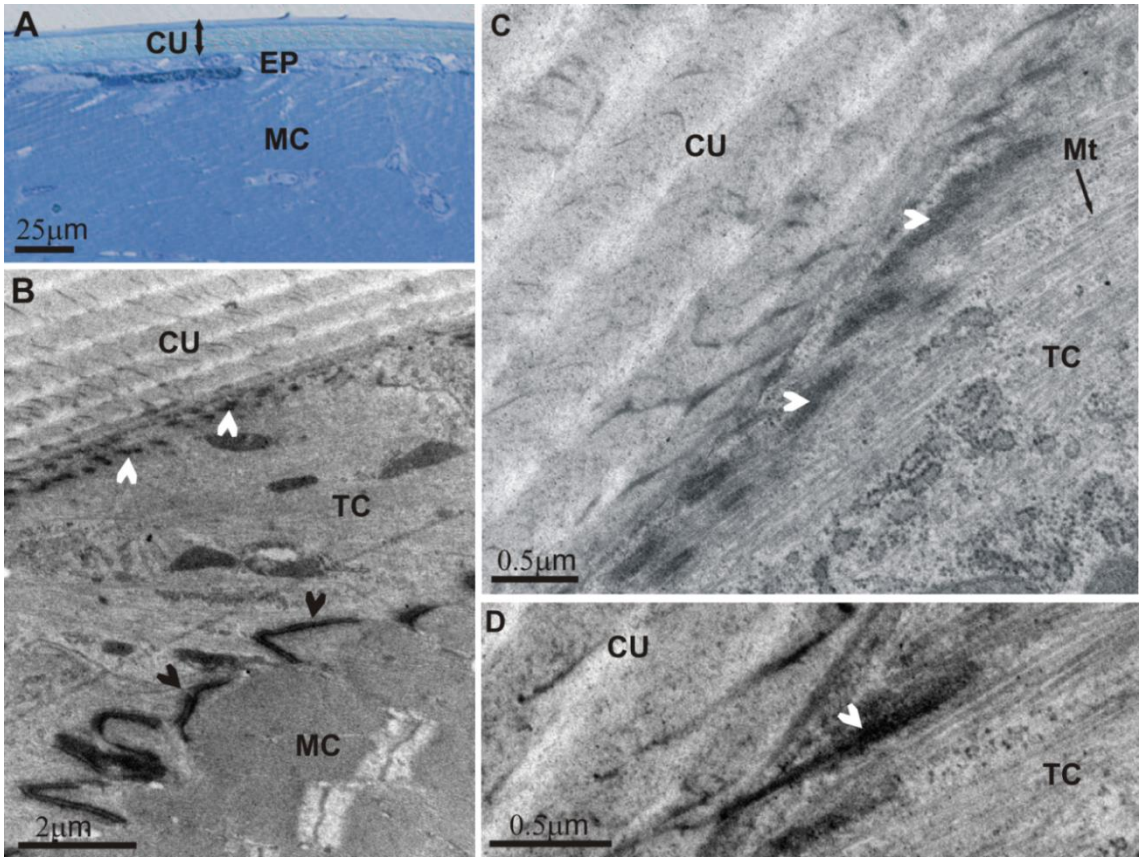
Supplementary figure

Exoskeleton-muscle attachment in the adult Ligia italica with the entire cuticle of the usual thickness. (doi: 10.3897/zookeys.176.2445.app) File format: Portable (Public) Network Graphic (png).

Citation: Žnidaršič N, Mrak P, Tušek-Žnidarič M, Štrus J (2012) Exoskeleton anchoring to tendon cells and muscles in molting isopod crustaceans. In: Štrus J, Taiti S, Sfenthourakis S (Eds) Advances in Terrestrial Isopod Biology. ZooKeys 176: 39–53. doi: 10.3897/zookeys.176.2445.app



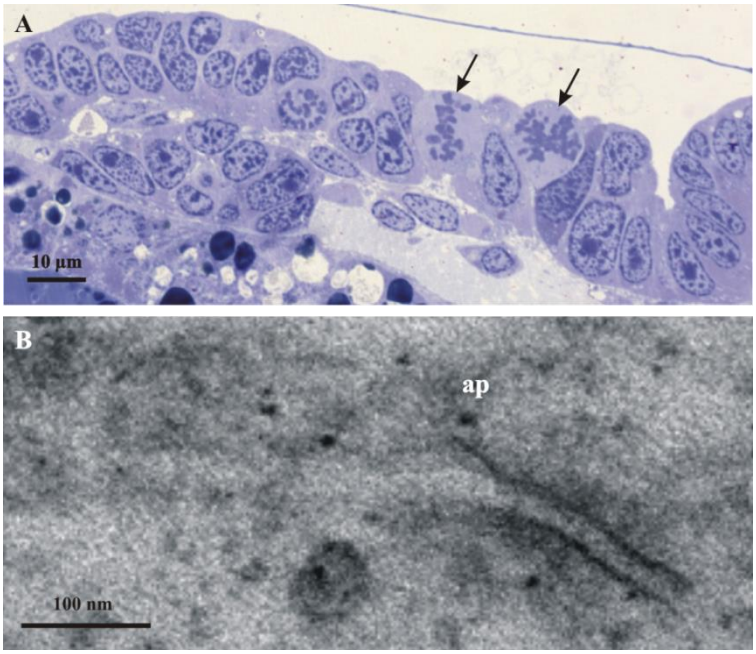
Supplementary figure:
Exoskeleton-muscle attachment in the adult *Ligia italica* with the entire cuticle of the usual thickness. The overall general architecture of the exoskeleton-muscle attachment in this isopod crustacean is similar to that in other arthropods (A a semithin section, B). Muscle cells are connected to specialized epidermal cells, tendon cells, by myotendinous junctions. Apically, the tendon cells are connected to the exoskeleton (cuticle) by several apical anchoring junctions (C, D). CU cuticle EP epidermis MC muscle cells Mt microtubules TC tendon cells; black arrowheads – myotendinous junctions; white arrowheads – apical anchoring junctions.

2.2 DRUGO POVEZOVALNO ZNANSTVENO DELO

2.2.1 Ultrastruktura epidermisa pri srednjih embrijih, poznih embrijih in marzupijskih mankah mokrice *P. scaber*

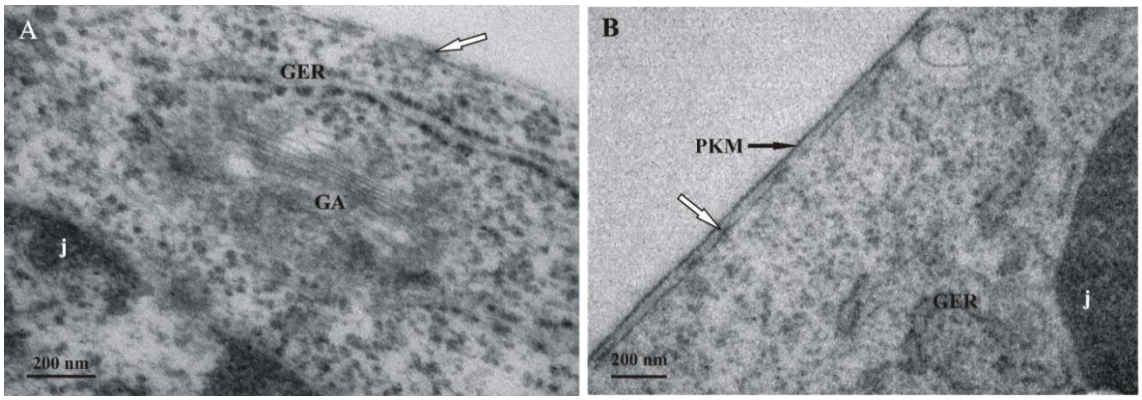
Pri analizi ultrastrukture epidermalnih celic smo se osredotočili na značilnosti, ki so predvidoma povezane s celično diferenciacijo in s sintezo zunajceličnega matriksa, kot so prisotnost medceličnih stikov, modifikacije apikalne plazmaleme in organiziranost apikalne citoplazme. V ta namen smo uporabili vzorce embrijev in marzupijskih mank, ki so bili analizirani v študiji diferenciacije eksoskeletne kutikule vrste *P. scaber* (poglavje 2.1.3). Proučili smo: štiri stadije srednjega embrija (stadiji 10, 13, 14 in 15), tri stadije poznega embrija (stadiji 16, 18 in 19) in štiri stadije marzupijske manke (novo izležena zgodnja marzupijska manka, napredna zgodnja marzupijska manka, srednja marzupijska manka in pozna marzupijska manka).

Pri srednjem embriju v stadiju 10, pri katerem smo opazili zgodnje znake izločanja apikalnega zunajceličnega matriksa epidermisa, se epidermalne celice še intenzivno delijo. V subapikalni regiji celic so jasno razvidni medcelični adherentni stiki (sl. 8), ki mehansko povezujejo sosednji celici. Na mestu stika sta pod membranama obeh celic vidna sloja gostega materiala. Subapikalni adherentni stiki so bili v tem stadiju razvidni na prerezih vseh epidermalh celic, ki smo jih analizirali. Pri nekaterih stikih je citoplazemski sloj manj obsežen in manj elektronsko gost v primerjavi z drugimi (sl. 8). Pri stadiju 13 je epidermis urejen v enojni sloj celic cilindričnih do kubičnih oblik, na površini epidermisa pa je prisotna neprekinjena elektronsko gosta lamina zgodnjega prekutikularnega matriksa. Pri srednjih embrijih v stadijih 10 in 13, smo v apikalni citoplazmi epidermalnih celic opazili posamezne daljše cisterne granuliranega endoplazemskega retikuluma (GER) in nekaj mitohondrijev, redkeje pa so v regiji med jedrom in apikalno plazmalemo prisotne tudi posamezne skladovnice Golgijevega aparata, sestavljene iz treh do štirih ploščatih cistern (sl. 9). Apikalna plazmalema tvori nizke izbokline, nad katerimi leži sloj rahlega materiala ali pa akumulacije elektronsko gostega materiala.



Slika 8: Epidermis in medcelični stiki pri srednjem embriju v stadiju 10. **A:** V epidermisu so opazne celične delitve (→). **B**: Adherentni stik iz manj obsežnega citoplazemskega materiala pod membranama obeh celic v subapikalni regiji. ap – apikalna plazmalema.

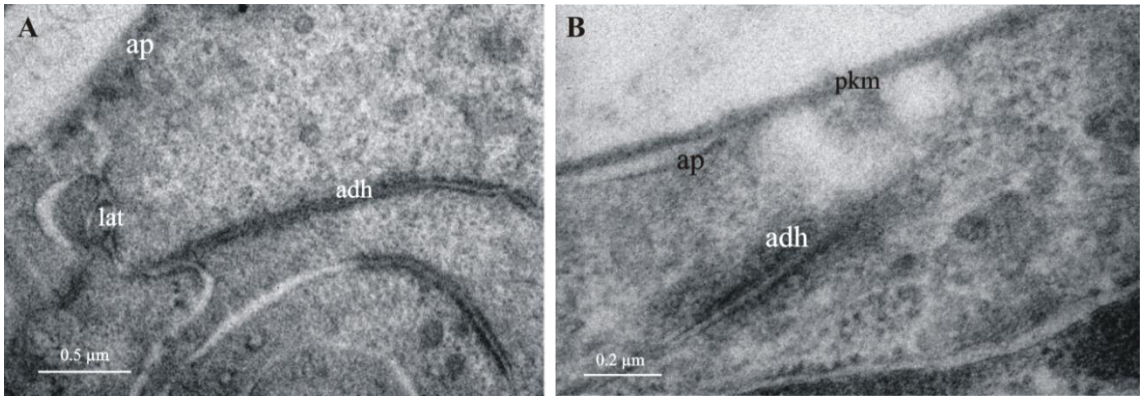
Figure 8: Epidermis and cellular junctions of mid-stage embryo in stage 10. **A**: Cell divisions are observed in the epidermis (→). **B**: Adherens junction, consisting of less abundant cytoplasmic material underneath both plasma membranes in the subapical region. ap – apical plasma membrane.



Slika 9: Ultrastruktura apikalnega predela epidermalnih celic srednjega embrija v stadiju 10 (**A**) in v stadiju 13 (**B**). Bela puščica: nizke izbokline apikalne plazmaleme, nad njimi je elektronsko gost material. Vidne so posamezne daljše cisterne granuliranega endoplazemskega retikuluma (GER). Redko so prisotne neizrazite skladovnice Golgijevega aparata (GA). PKM: zgodnji prekutikularni matriks.

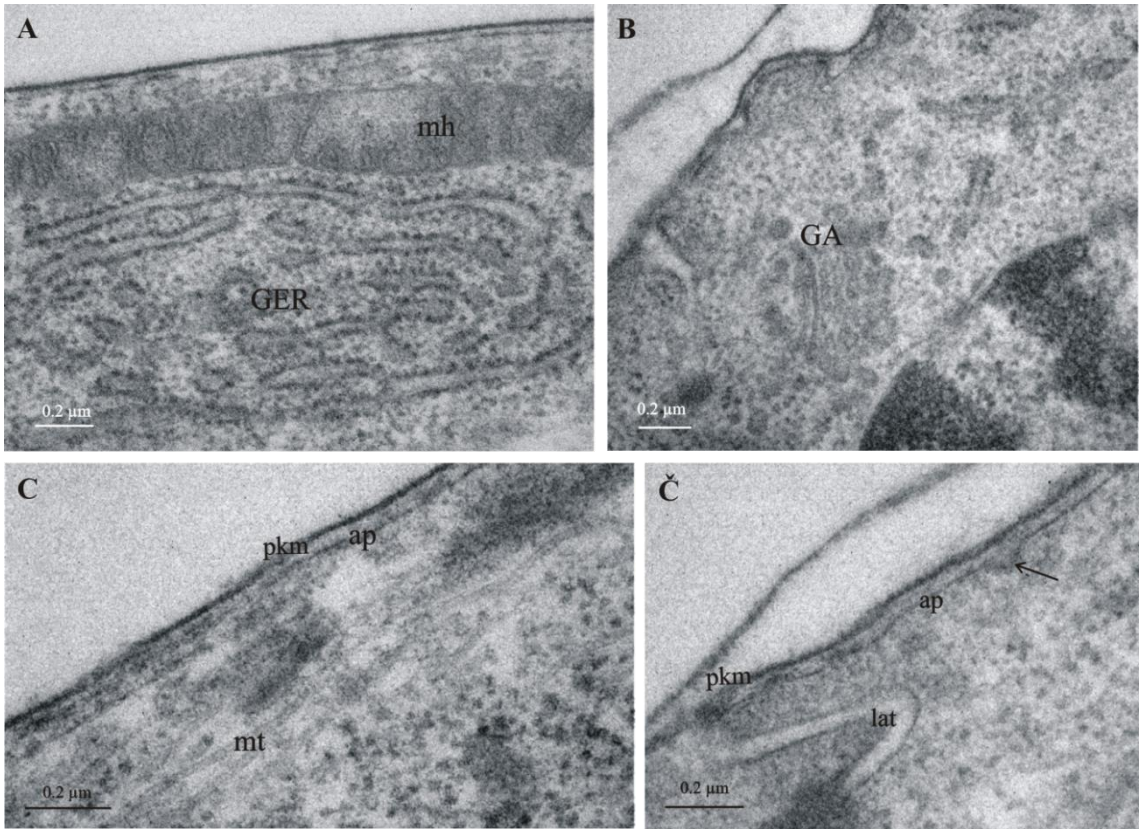
Figure 9: Ultrastructure of the apical region of epidermal cells: mid-stage embryo in stage 10 (**A**) and in stage 13 (**B**). White arrows: shallow bulges of apical plasma membrane with electron dense material. Single long cisterns of granular endoplasmic reticulum (GER) are evident. Stacks of Golgi apparatus (GA) are inconspicuous and rarely observed. PKM: early precuticular matrix.

V poznih stadijih srednjega embrija (stadija 14 in 15) in zgodnjem stadiju poznega embrija (stadij 16), pri katerih nastaja naslednji prekutikularni matriks, epidermis gradijo kubične do ploščate celice. Lateralni plazmalemi sosednjih celic sta v subapikalnem predelu pogosto nagubani in razvejani ter povezani z obsežnimi adherentnimi stiki, ki jih tvori elektronsko gost citoplazemski material pod obema membranama (sl. 10). Apikalna plazmalema epidermalnih celic v razvojnih stadijih 14, 15 in 16 je oblikovana v nizke izbokline, na konicah katerih je elektronsko gost material, nad njimi pa se raztezajo kratka elektronsko gosta vlakna (sl. 11). Granulirani ER je dobro razvit (sl. 11A), vidnih je veliko daljših cistern. Posamezni diktiosomi, skladovnice Golgijevega aparata, ter številni z njimi povezani vezikli so vidni v regiji med jedrom in apikalno plazmalemo (sl. 11B). Skladovnice so izrazite, sestavljene iz štirih do petih cistern, nekatere cisterne vsebujejo elektronsko gostejši material. Pod apikalno plazmalemo so pogosto prisotni elektronsko gosti vezikli, večkrat opazimo tudi vezikle, ki so zliti z apikalno plazmalemo (sl. 10, 11B in Č). Številni mikrotubuli so opazni pod apikalno celično površino (sl. 11C).



Slika 10: Ultrastruktura epidermalnih celic in medceličnih stikov pri embrijih v razvojnih stadijih 14, 15 in 16. Nad apikalno plazmalemo (ap) je viden prekutikularni matriks (pkm). Lateralne meje celic v subapikalnem predelu (lat) so zavite in razvejane (**A**). Celice so povezane z adherentnimi stiki (adh) (**A, B**).

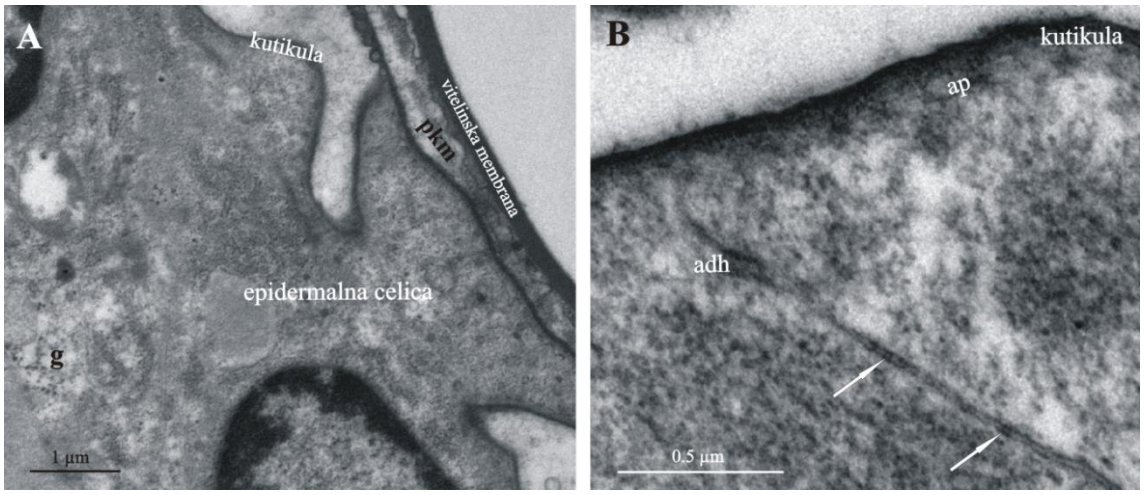
Figure 10: Ultrastructure of epidermal cells and cell junctions in embryonic stages 14, 15 and 16. Precuticular matrix (pkm) is evident above the apical plasma membrane (ap). Lateral cell boundaries in subapical region (lat) are undulated and branched (**A**). The cells are connected by adherens junctions (adh) (**A, B**).



Slika 11: Ultrastruktura apikalnega predela epidermalnih celic pri embrijih v razvojnih stadijih 14, 15 in 16. Nad apikalno plazmalemo (ap) je prisoten prekutikularni matriks (pkm). Apikalna citoplazma vsebuje dobro razvit GER, mitohondrije (mh) (**A**), posamezne skladovnice Golgijevega aparata (GA) (**B**) in elektronsko goste vezikle (**B**). Pod apikalno plazmalemo (ap) so vidni številni mikrotubuli (mt) (**C**). **Č:** Vezikel, zlit z apikalno plazmalemo (→). Lateralne celične membrane so zavite (lat).

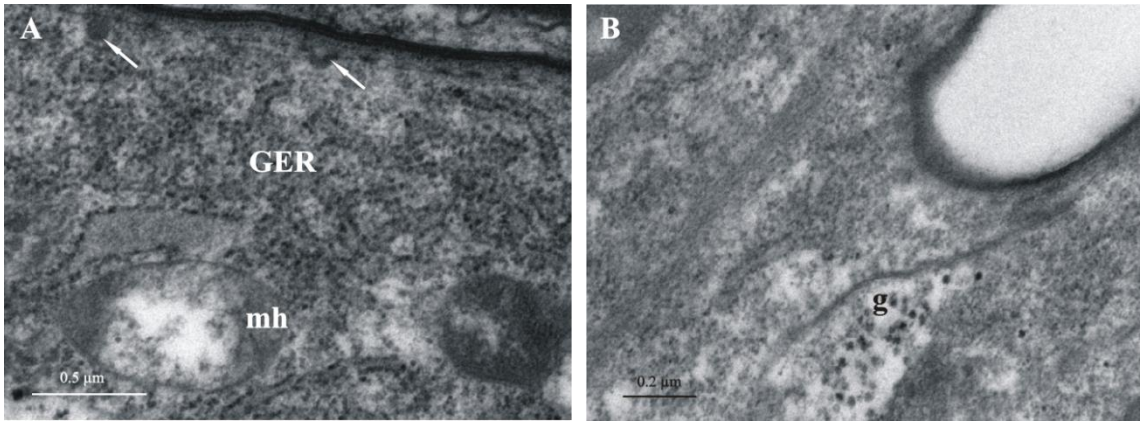
Figure 11: Ultrastructure of apical region of epidermal cell in embryonic stages 14, 15 and 16. Precuticular matrix (pkm) is evident above the apical plasma membrane (ap). Apical cytoplasm contains prominent GER, mitochondria (mh) (**A**), single stacks of Golgi apparatus (GA) (**B**) and electron dense vesicles (**B**). Numerous microtubules (mt) are evident underneath the apical plasma membrane (ap) (**C**). **Č:** A vesicle, fused with the apical plasma membrane (→). The lateral cell membranes are undulated (lat).

Pri poznem embriju pred izleganjem v stadiju 18, pri katerem je vidna tvorba prvega kutikularnega matriksa, so epidermalne celice sploščene, jedro pa je pomaknjeno na bazalni del. Apikalna celična površina je izrazito nagubana, kutikula pa se ji natančno prilega (sl. 12A). Sosednje celice so subapikalno povezane z adherentnimi stiki, bazalno pod njimi pa so v medceličnem prostoru pogosto vidne posamezne pregrade (pl. septa) (sl. 12B), za katere sklepamo, da so prva septa v formaciji septiranih medceličnih stikov. Apikalna plazmalema epidermalnih celic je oblikovana v nizke izbokline s plaki, nad katerimi je prisoten nastajajoči kutikularni matriks (sl. 12B, 13A). Citoplazma epidermalnih celic poznih embrijev v stadiju 18 je gosta. Izrazita ultrastrukturna značilnost je obsežen granulirani ER predvsem v apikalnem delu celice, veliko pa je tudi mitohondrijev. Razširjene cisterne GER so pogosto v tesnem stiku z mitohondriji (sl. 13A). Podobno kot pri prejšnjih opisanih stadijih tudi v tem stadiju pogosto opazimo elektronsko goste vezikle in vezikle, zlite z apikalno plazmalemo (sl. 13A). Območja akumulacij glikogenskih zrnc so vidna predvsem na bazalnem delu celice in tik ob lateralni plazmalemi (sl. 12A, 13B). Med gosto citoplazmo apikalno nad jedrom lahko opazimo snope mikrotubulov, orientiranih vzporedno ali diagonalno na apikalno plazmalemo.



Slika 12: Ultrastruktura epidermalnih celic v embrionalnem stadiju 18. **A**: Nastajajoča kutikula se natančno prilega nagubani apikalni površini celic. Akumulacije glikogenskih zrnc (g) so prisotne na bazalnem delu celice. **B**: Sosednje celice so apikalno povezane z adherentnimi stiki (adh), v medceličnem prostoru pod njimi pa so vidne posamezne pregrade (bele puščice), ki verjetno predstavljajo začetek tvorbe septiranih stikov. ap: apikalna plazmalema; pkm: prekutikularni matriks.

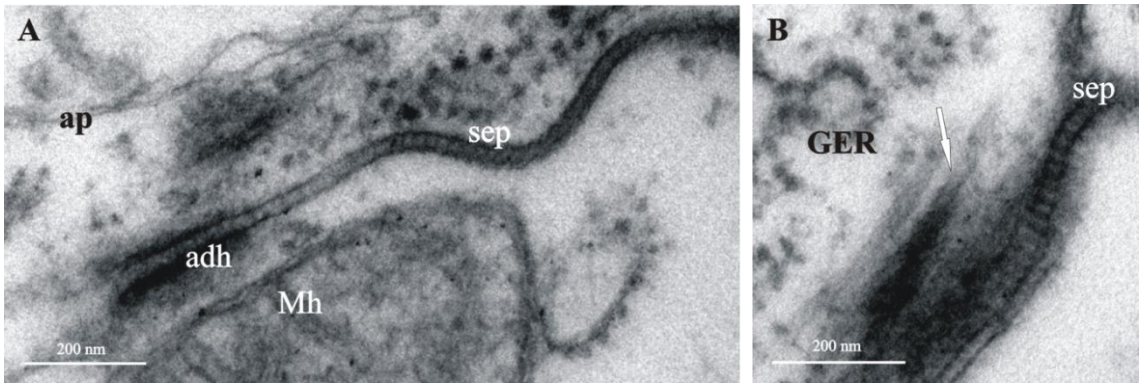
Figure 12: Ultrastructure of epidermal cells in embryonic stage 18. **A**: The newly forming cuticle is strictly aligned with the outline of ruffled apical cell surface. Accumulation of glycogen granules (g) are evident in basal region of the cell. **B**: Adjoining cells are apically connected by adherens junctions (adh). Single septa are visible in the intercellular space basally beneath adherent junctions (white arrows), probably representing the beginning of septate junction formation. ap: apical plasma membrane; pkm: precuticular matrix.



Slika 13: Ultrastruktura apikalnega predela epidermalnih celic pri poznem embriju v stadiju 18. **A**: Apikalna citoplazma je zapolnjena z granuliranim ER (GER). Razširjena cisterna GER v bližini mitohondrija (mh). Bele puščice: Elektronsko gost vezikel tik pod apikalno plazmalemo in vezikel zlit s plazmalemo. **B**: Akumulacija glikogenskih zrnc (g) ob lateralni plazmalemi.

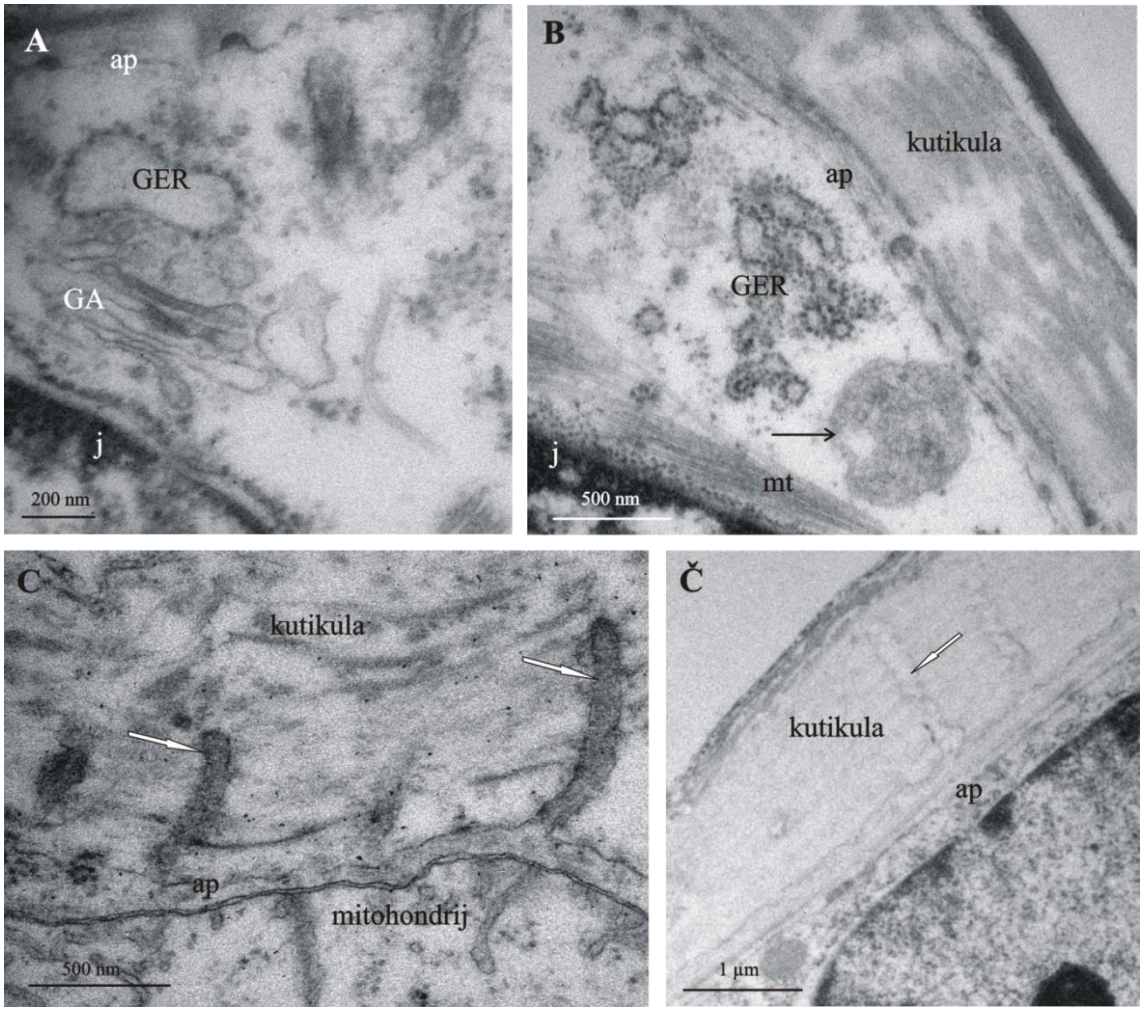
Figure 13: Ultrastructure of apical region of epidermal cells in prehatching embryo of stage 18. **A**: The apical cytoplasm is filled with granular ER (GER). A broad GER cistern in the proximity of the mitochondrium (mh). White arrows: Electron dense vesicle under the apical plasma membrane and vesicle fused with the plasma membrane. **B**: Accumulation of glycogen granules (g) by the lateral plasma membrane.

V zadnjem stadiju embriogeneze (stadij 19), ko je embrij med oziroma tik pred izvalitvijo iz vitelinske membrane, je eksoskeletna kutikula debelejša in diferencirana v tri glavne kutikularne sloje. Epidermalne celice so ploščate, njihova površina pa ni izrazito nagubana. Pod adherentnimi stiki pogosto opazimo daljše neprekinjeno zaporedje sept v medceličnem prostoru, ki sestavlja septirani stik z lestvičastim izgledom (sl. 14A). Citoplazma epidermalnih celic v zadnjem embrionalnem stadiju (stadij 19) je precej redka. Mitohondriji so številni in veliki, z izrazitimi kristami. Tik ob jedru v smeri proti apikalni plazmalemi lahko opazimo posamezne diktiosome, skladovnice Golgijevih cistern, ki so obdane z vezikli. Nekatere cisterne vsebujejo tudi elektronsko gostejši material (sl. 15A). Granulirani ER je prisoten pretežno v apikalnem predelu celice, pogoste so razširjene cisterne (sl. 15B). Številni mikrotubuli potekajo skoraj vzporedno z apikalno plazmalemo (sl. 15B). Na apikalni plazmalemi so poleg kratkih izboklin lahko prisotni dolgi citoplazemski izrastki, ki segajo globoko v odmaknjeno kutikulo in vsebujejo elektronsko gostejši material (sl. 15C). Od epidermisa rahlo odmaknjena kutikula je tako v tem stadiju še vedno v tesni povezavi s celicami. V apikalni citoplazmi smo opazili tudi heterogene vezikularne strukture, podobne multivezikularnim telesom (sl. 15B). Pri embriju z bolj razgrajeno kutikulo, diferenciacije apikalne plazmaleme niso izrazite, so pa v odmaknjeni kutikuli prisotni porni kanali, ki ne vsebujejo elektronsko gostih citoplazemskih izrastkov (sl. 15Č).



Slika 14: Ultrastruktura medceličnih stikov v epidermisu poznega embrija v stadiju 19. **A**: Adherentni stik (adh) se nahaja pod apikalno plazmalemo (ap), pod njim pa je oblikovan septirani stik (sep). Mh: mitohondrij. **B:** Na stik je pritrjen citoskelet (bela puščica). GER: granulirani endoplazemski retikulum.

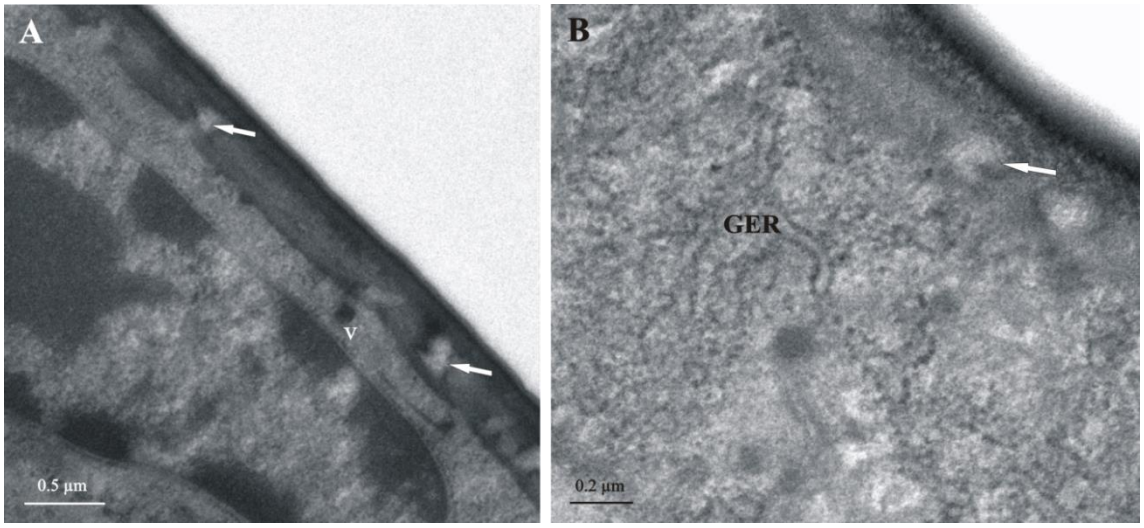
Figure 14:Ultrastructure of cell junctions in the epidermis of late embryo in stage 19. **A**: Adherens junction (adh) is present below apical plasma membrane (ap). Septate junction (sep) is formed under adherens junction. Mh: mitochodrium. **B**: Cytoskeletal elements are connected to the junction (white arrow). GER: granular endoplasmatic reticulum.



Slika 15: Ultrastruktura apikalnega predela epidermalnih celic pri poznem embriju v stadiju 19. **A**: Diktiosom Golgijevega aparata (GA) in GER se nahajata med jedrom (j) in apikalno plazmalemo (ap). Ostala citoplazma je precej redka. **B**: V apikalni citoplazmi so prisotni skupki granuliranega ER s razširjenimi cisternami (GER) in heterogena struktura, podobna multivezikularnemu telesu (→). Snop mikrotubulov (mt) leži apikalno tik ob jedru in je orientiran diagonalno na apikalno plazmalemo (ap). **C**: Dolgi citoplazemski izrastki segajo globoko v odmaknjeno kutikulo in vsebujejo elektronsko gost material (bele puščice). **Č**: Pri embriju z bolj razgrajeno kutikulo so v njej prisotni porni kanali brez elektronsko gostih citoplazemskih izrastkov (bela puščica).

Figure 15: Ultrastructure of epidermal cell apical region of late embryo in stage 19. **A**: Dictyosome of Golgi apparatus (GA) and granular ER (GER) are present between nucleus (j) and apical plasma membrane (ap). The rest of the cytoplasm is scarce. **B**: Clusters of granular ER with broad cisterns (GER) and a heterogenous structure, that resemble a multivesicular body (→), are present in the apical cytoplasm. A bundle of microtubules (mt) lies apically by the nucleus and is oriented diagonally to apical plasma membrane (ap). **C**: Long cytoplasmic projections, containing electron dense material, extend deep into the detached cuticle (white arrows). **Č**: In the embryo with more degraded cuticle, pore canals, lacking electron dense cytoplasmic projections, are present in the cuticle.

Pri novo izleženi marzupijski manki so epidermalne celice pokrite s tanko kutikulo, ki je diferencirana v epi- in prokutikulo in se natančno prilega nagubani površini celic. V prokutikulo segajo številni široki citoplazemski izrastki (sl. 16A in B). Pod apikalno plazmalemo se pogosto nahajajo elektronsko gosti vezikli (sl. 16A), prisotno pa je tudi obsežno omrežje sploščenih cistern GER (sl. 16B). Skladovnic Golgijevih cistern nismo opazili in na apikalni plazmalemi ni razvidnih plakov.



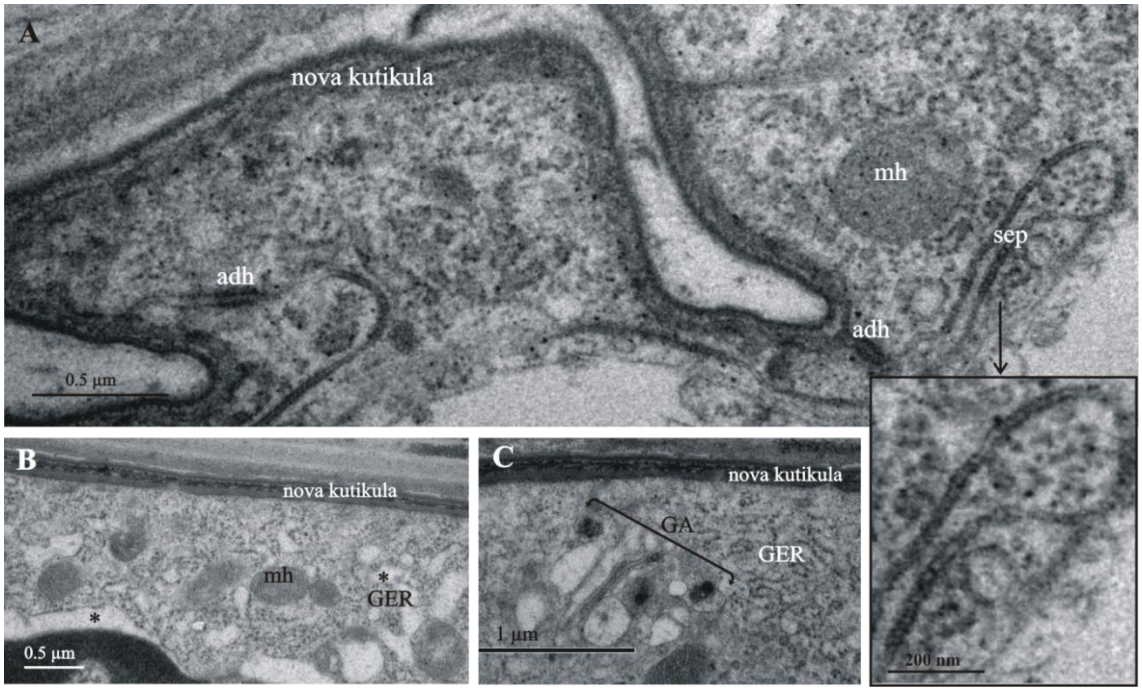
Slika 16: Ultrastruktura epidermalnih celic pri novo izleženi marzupijski manki. V prokutikulo segajo številni široki citoplazemski izrastki (bele puščice). V apikalni citoplazmi opazimo elektronsko goste vezikle (v) in zlivanje veziklov z plazmalemo **(A)**, ter obsežno omrežje sploščenih cistern GER **(B)**.

Figure 16: Ultrastructure of epidermal cells in newly hatched marsupial manca. Broad cytoplasmic projections extend into the procuticle (white arrows). Electron dense vesicles (v) and vesicle fusion with plasma membrane (**A**) and extensive network of flat GER cisterns (**B**) are observed in the apical cytoplasm.

V nadaljnjem razvoju marzupijske manke, pri napredni zgodnji manki in srednji manki, pri katerih je prokutikula debelejša in diferencirana v izraziti eksokutikulo in endokutikulo, epidermalne celice vsebujejo številne mitohondrije, elektronsko goste vezikle in mikrotubule. V nekaterih vzorcih so prisotni citoplazemski izrastki, ki segajo v kutikulo, in izbokline apikalne plazmaleme, kar je znak izločanja kutikularnega matriksa.

Pri poznih marzupijskih mankah, pri katerih nastaja nova kutikula, so epidermalne celice kubične do ploščate oblike. Apikalna celična površina je izrazito nagubana, apikalna plazmalema pa je oblikovana v nizke izbokline s plaki. Medcelični stiki so diferencirani v kratke adherentne stike, ki so nameščeni v subapikalnem predelu, in septirane stike, ki zavzemajo precejšen del lateralnih membran (sl. 17A). Apikalna citoplazma je zapolnjena s celičnimi organeli. Granulirani ER se razprostira po celotni

citoplazmi. Perinuklearni prostor in svetlina GER sta pogosto razširjena (sl. 17B). Golgijev aparat je dobro razvit in pogost (sl. 17C). Diktiosomi so prisotni v regiji apikalno od jedra, pod nastajajočo kutikulo. Posamezni diktiosomi so navadno sestavljeni iz nekaj medianih sploščenih cistern, ki so na robovih razširjene, svetlina *trans*- in *cis*-cistern pa je pogosto v celoti razširjena. Okrog Golgijevega aparata so številni vezikli, nekateri imajo elektronsko gosto vsebino. Pogosto so pod apikalno plazmalemo prisotni elektronsko gosti vezikli. Mitohondriji so številni. Mikrotubuli potekajo v različnih smereh, v apikalno-bazalni osi in pod apikalno plazmalemo vzporedno s površino celice.



Slika 17: Ultrastruktura apikalnega predela epidermalnih celic pri pozni marzupijski manki. **A**: Površina celic in tanka nova kutikula nad njo sta izrazito nagubani. Celice so povezane s kratkimi adherentnimi stik tik pod apikalno plazmalemo (adh), bazalno pod njimi pa z dolgimi septiranimi stiki (sep). **B**: Obširen granulirani ER (GER) in številni mitohondriji (mt) so prisotni v apikalni citoplazmi. Na nekaterih predelih je opazna razširjena svetlina GER in razširjen perinuklearni prostor (*). **C**: Skladovnica Golgijevega aparat (GA) se nahaja apikalno od jedra (na sliki ni viden), obrnjena proti apikalni plazmalemi. Nekateri predeli GA in nekateri okoliški vezikli vsebujejo elektronsko gost material.

Figure 17: Ultrastructure of apical region of epidermal cells in late marsupial manca. **A**: Cell surface and overlying thin newly forming cuticle are ruffled. Cells are interconnected by short adherens junctions immediately under the apical membrane (adh) and long septate junctions basally beneath them (sep). **B**: Extensive granular ER (GER) and numerous mitochondria are present in the apical cytoplasm. Broad lumen of GER and broad perinuclear space are observed in some regions (*). **C**: Golgi apparatus stack (GA) is present apically to the nucleus (not shown in the image) and oriented towards the apical membrane. Several GA regions and surrounding vesicles contain electron dense material.

2.2.2 Ultrastruktura kutikularnega matriksa v črevesu med razvojem mokrice *P. scaber* v valilniku

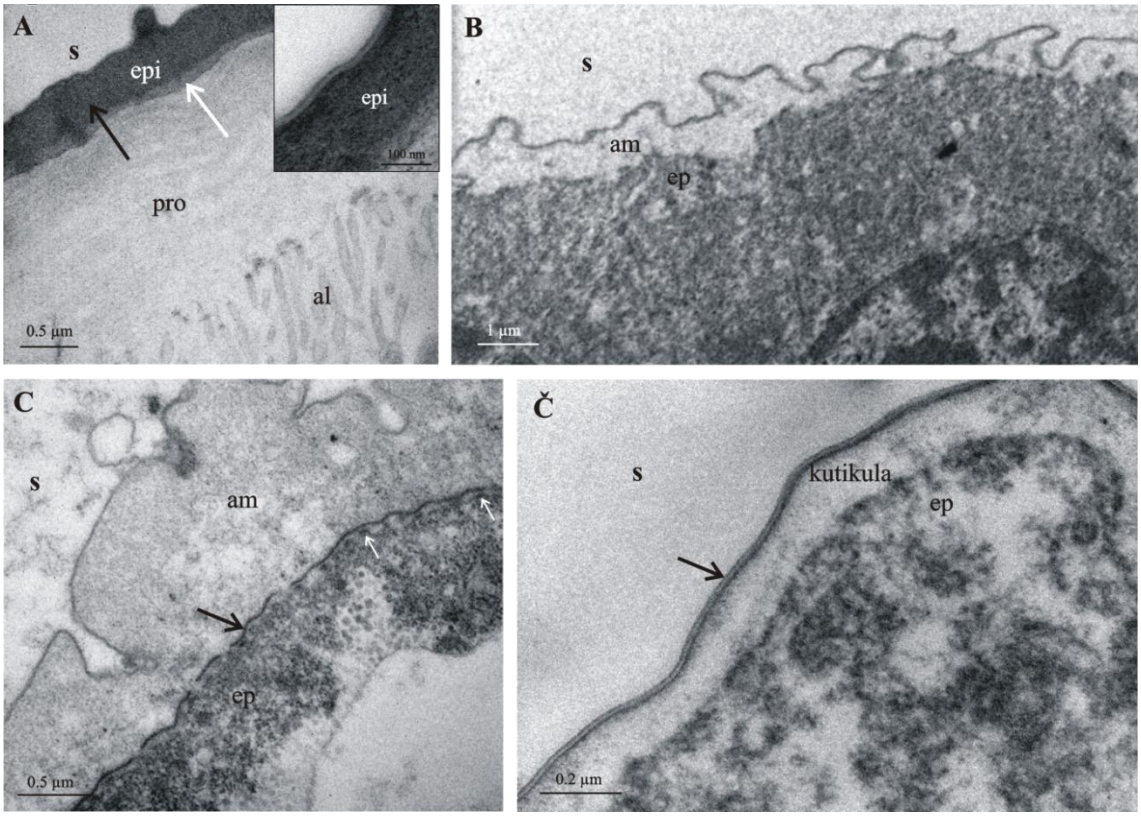
Analizirali smo ultrastrukturo apikalnih matriksov epitelnih celic zadnjega črevesa v izbranih razvojnih stadijih embrijev in ličink v valilniku mokrice *P. scaber* in jo primerjali z ultrastrukturo črevesne kutikule odraslih živali. V ta namen smo uporabili vzorce embrijev in marzupijskih mank, ki so bili analizirani v študiji diferenciacije eksoskeletne kutikule vrste *P. scaber* (poglavje 2.1.3), in sicer naslednje stadije: tri stadije poznega embrija (stadiji 16, 18 in 19) in dva stadija marzupijske manke (zgodnja marzupijska manka in pozna marzupijska manka). Odrasle osebke brez opaznih znakov levitve smo izbrali iz laboratorijske kulture, iz njih izolirali črevo in vzorce fiksirali v 2.5 % glutaraldehidu v 0.1 M pufru Hepes. Sledil je enak postopek postfiksacije in vklapljanja v smolo kot pri vzorcih embrijev in mank (Bogataj, 2014). Zadnje črevo odraslih živali je razdeljeno na tri osnovne dele: anteriorno komoro, ki sega od želodca do približno polovice zadnjega črevesa, papilatno regijo, ki sega od anteriorne komore do sfinktra in rektum, ki se konča z anusom. Ultrastrukturno smo analizirali dva predela zadnjega črevesa: anteriorno komoro in papilatno regijo.

Pri odrasli živali je kutikula zadnjega črevesa debela nekaj μm in sestavljena iz zunanje elektronsko goste epikutikule in notranje elektronsko svetlejše prokutikule (sl. 18A). V površinskem predelu epikutikule razločimo dva do tri tanke sloje, pod katerimi sta debela elektronsko gosta plast in tanjša nekoliko svetlejša plast. V anteriorni komori so v prokutikuli, ki je nekajkrat debelejša od epikutikule, vidne lamele hitinsko-proteinskih vlaken (sl. 18A). V papilatni regiji sta epi- in prokutikula približno enake debeline, prokutikula je fibrilarne strukture, lamele pa niso opazne (Mrak in sod., 2013; Bogataj, 2014).

V zadnjem črevesu poznih embrijev v stadijih 16 in 18 je prisoten apikalni zunajcelični matriks z izrazito nagubano površino (sl. 18B, C). Sestavljen je iz elektronsko svetlega materiala brez vidnih podslojev, ki ga prekriva elektronsko gosta lamina. V stadiju 18 je poleg prekutikularnega matriksa tik nad apikalno plazmalemo črevesnih celic prisoten novo nastajajoči matriks, ki se prilega apikalni površini celic (sl. 18C). Sestavlja ga tanek elektronsko gost sloj, ki je ponekod še prekinjen. Kutikularni trni še niso opazni. Pri teh vzorcih je apikalna plazmalema črevesnih celic oblikovana v kratke izbokline z elektronsko gostimi konicami. V zadnjem stadiju embriogeneze (stadij 19), ko je embrij blizu izleganja iz vitelinske membrane, je apikalni matriks črevesnih celic sestavljen iz troslojne distalne lamine, elektronsko goste plasti pod njo in notranjega debelega elektronsko svetlega sloja.

V zgodnjih stadijih marzupijske manke je v črevesu oblikovana kutikula, ki leži tesno nad epitelom. Debelina kutikule znaša med 0.1 μm in 0.6 μm. Strukturno je podobna v

obeh regijah zadnjega črevesa, sestavljena iz homogene elektronsko svetle prokutikule in nad njo ležeče tanke elektronsko goste epikutikule, katere površinski del je iz treh tankih slojev (sl. 18Č). V anteriorni komori ni prepoznavnih podslojev prokutikule. Pri poznih marzupijskih mankah je elektronsko gosta plast epikutikule pod površinskim troslojem nekoliko debelejša kot pri zgodnejših ličinkah. V poznih marzupijskih stadijih so ponekod na površini črevesne kutikule diferencirani kutikularni trni, struktura kutikule v različnih regijah zadnjega črevesa pa je še vedno podobna. Opazni so tudi znaki levitve kutikule, kot so odmik črevesne kutikule od epitela, razgradnja kutikularnih notranjih slojev in sinteza nove kutikule nad celicami.



Slika 18: Ultrastruktura apikalnih matriksov zadnjega črevesa raka enakonožca *P. scaber* pri odraslem (**A**), pri poznem embriju v stadiju 16 (**B**), pri poznem embriju v stadiju 18 (**C**) in pri zgodnji marzupijski manki (**Č**). s – svetlina črevesa. **A**. Kutikula anteriorne komore odrasle živali je sestavljena iz zunanje elektronsko goste epikutikule (epi) in notranje debelejše prokutikule s podsloji hitinsko-proteinskih vlaken (pro). V površinskem delu epikutikule razločimo dva do tri tanke sloje (vstavljena slika), pod njimi pa sta dve homogeni plasti: debela elektronsko gosta plast (črna →) in tanjša svetlejša plast (bela →). Apikalna plazmalema celice črevesnega epitela je nagubana v apikalni labirint (al). **B.** Pri poznem embriju v stadiju 16 je nad epitelnimi celicami zadnjega črevesa (ep) prisoten apikalni zunajcelični matriks z izrazito nagubano površino (am). Sestavljen je iz elektronsko svetlega materiala brez vidnih podslojev in tanke elektronsko goste lamine. **C**. Pri poznem embriju v stadiju 18 je apikalni zunajcelični matriks (ap) črevesnih epitelnih celic (ep) obsežen in naguban. Apikalna plazmalema epitelne celice je oblikovana v nizke izbokline z elektronsko gostimi konicami (beli →), tik nad njimi pa je prisoten tanek elektronsko gost sloj novo nastajajočega matriksa (črna →), ki pa je ponekod še prekinjen. **Č**. Pri zgodnji

marzupijski manki tesno nad epitelno celico (ep) leži kutikula, ki je vzdolž celotnega zadnjega črevesa sestavljena iz tanke elektronsko goste epikutikule iz treh tankih slojev (→) in elektronsko svetle prokutikule brez razvidnih podslojev.

Figure 18: Ultrastructure of apical matrices in the hindgut of *P. scaber* adult (**A**), late embryo in stage 16 (**B**), late embryo in stage 18 (**C**) and early marsupial manca (**Č**). s – lumen of the gut. **A**. The cuticle in the anterior chamber of adult animal consists of the outer electron dense epicuticle (epi) and the inner thicker procuticle with sublayers of chitin-protein fibers (pro). Two to three layers are visible in the superficial part of the epicuticle (insert) and under them there are two homogenous layers: thick electron dense layer (black →) and thinner and more lucent layer (white →). Apical plasma membrane of epithelial cell forms apical labyrinth (al). **B**. Late embryo in stage 16: Epithelial cells of the hindgut (ep) are covered by an apical extracellular matrix with intensely ruffled surface (am). The matrix consists of an electron lucent material with no distinct sublayers and a thin electron dense lamina. **C**. Late embryo in stage 18: The apical extracellular matrix (ap) of epithelial cells (ep) is extensive and ruffled. Apical plasma membrane of epithelial cell forms shallow bulges with electron dense tips (white →). A thin electron dense layer of a newly forming matrix (black →) is observed apposed to the bulges and is still in fragments. **Č**. Early marsupial manca: The cuticle is closely apposed to the apical surface of epithelial cell (ep). The cuticle along the whole hindgut consists of a thin electron dense three-layered epicuticle (→) and an electron lucent procuticle with no distinct sublayers.

3 RAZPRAVA IN SKLEPI

3.1 RAZPRAVA

3.1.1 Ultrastruktura jajčnih ovojnic mokrice iz rodu *Porcellio*

Jajčne ovojnice členonožcev so specializirani matriksi, ki obdajajo jajčno celico in primarno zaščitijo embrije med razvojem pred izsušitvijo in osmotskim stresom. Ultrastrukturo jajčnih ovojnic raka enakonožca rodu *Porcellio* smo primerjali z modelnim nevretenčarskim organizmom *Drosophila melanogaster*, kopenskim členonožcem, ki ima drugačne strategije razvoja na kopnem. Rezultati so pokazali, da so jajčne ovojnice embrijev mokrice tanjše in manj kompleksne v primerjavi z jajčnimi ovojnicami zrelih jajčec iz ovarijev vinske mušice (Margaritis in sod., 1980).

Horion embrijev mokric je približno dvakrat tanjši v primerjavi s horionom jajčec vinske mušice. Sestavljen je iz enega samega homogenega sloja, medtem ko je horion vinske mušice diferenciran v več slojev: eksohorion, endohorion in notranji horionski sloj. V horionskem matriksu mokrice so elektronsko svetle lakune, dolžine približno 200 nm in širine približno 100 nm. V endohorionskem sloju vinske mušice so obsežni prazni prostori, ki naj bi vsebovali zrak in s tem pospeševali izmenjavo dihalnih plinov ter imeli vlogo pri respiraciji (Margaritis in sod., 1980; Waring, 2000). Funkcija teh lakun v horionskem matriksu pri mokrici ni poznana. V študiji tolerance embrijev na fiziološki stres pri kopenskem raku enakonožcu vrste *Armadillidium vulgare* so ugotovili, da ima horion nizko prepustnost za vodo in raztopine in prispeva k visoki toleranci zgodnjih embrijev na osmotski stres (Surbida in Wright, 2001).

Vitelinska membrana mokrice je strukturno podobna vitelinski membrani vinske mušice, vendar je tanjša. Pretežni del vitelinske membrane je obsežen sloj iz homogenega matriksa, nad njim je tanek elektronsko gost sloj in tanek elektronsko svetel sloj z nagubano površino. Sestave vitelinske membrane nismo analizirali. Pri vinski mušici opisujejo tanek voskasti sloji med horionom in vitelinsko membrano, ki preprečuje izgubo vode (Margaritis in sod., 1980). Pri embrijih mokrice ne pričakujemo posebnih slojev s to vlogo, saj embriji niso izpostavljeni nevarnosti izgube vode.

Razlike v zgradbi jajčnih ovojnic rakov enakonožcev v primerjavi z žuželkami so pričakovane, saj embrionalni razvoj pri teh dveh skupinah kopenskih členonožcev poteka v različnih okoljih. Embriji rakov enakonožcev se razvijajo v vodnem okolju valilnika samice, napolnjega z marzupijsko tekočino, medtem ko so embriji vinske mušice izpostavljeni spreminjajočim razmeram v zunanjem okolju. Kompleksno zgrajene jajčne ovojnice pri vinski mušici, zlasti horion, so zato edina zaščitna pregrada med embrijem in kopenskim okoljem. Strukturno preprostejše in tanjše jajčne ovojnice

rakov enakonožcev pa sicer do določene mere ohranijo funkcijo nadzora embrijskega okolja, glavno vlogo zaščite pa prevzame zaprto okolje valilnika.

3.1.2 Ultrastruktura epidermalnih matriksov in epidermisa pri embrijih in marzupijskih mankah mokrice *P. scaber*

Sinteza in izločanje apikalnih zunajceličnih matriksov potekata že med embrionalnim razvojem in sta povezana z diferenciacijo epitelnih celic. V nalogi smo proučili tvorbo in diferenciacijo apikalnih zunajceličnih matriksov epidermisa s strukturnega vidika in ultrastrukturne značilnosti epidermalnih celic med embrionalnim in larvalnim razvojem znotraj valilnika pri raku enakonožcu *P. scaber*. V ta namen smo analizirali štiri stadije srednjega embrija, tri stadije poznega embrija in štiri stadije marzupijske ličinke manke. Embrionalne stadije smo določili v skladu s klasifikacijskim sistemom razvoja *P. scaber* v valilniku (Milatovič in sod., 2010), ki opisuje dvajset morfološko različnih zaporednih razvojnih stadijev: 19 embrionalnih stadijev in marzupijsko ličinko manko. Glede na morfološke spremembe, ki se dogajajo med razvojem marzupijske manke (rast manke, zmanjševanje količine rumenjaka v prebavnih žlezah, povečanje pigmentacije integumenta in povečano gibanje manke), smo na novo določili tri osnovne stadije manke: zgodnja, srednja in pozna marzupijska manka. Prehod iz embrija v manko vključuje večje morfološke spremembe in glede na podatke iz literature tudi osmoregulacijske spremembe. Predvidevali smo, da se bo to odražalo tudi na spremembah eksoskeletne kutikule, zato smo zgodnje marzupijske manke dodatno klasificirali v dva podstadija: novo izležena zgodnja manka in napredna zgodnja manka.

3.1.2.1 Diferenciacija prekutikularnih matriksov in ultrastruktura epidermisa pri srednjih in poznih embrijih

Z izrazom 'prekutikularni matriks' označujemo zunajcelični matriks, ki ga izloča embrionalni epidermis pred formacijo kutikule in se strukturno razlikuje od tipične eksoskeletne kutikule členonožcev. Najbolj očitna razlika je, da ni diferenciran v značilne horizontalne sloje. Naši rezultati kažejo, da se v drugi polovici embriogeneze raka enakonožca *P. scaber* izločita vsaj dva prekutikularna matriksa. Najzgodnejša embrionalna faza, pri kateri smo opazili znake izločanja epidermalnega matriksa, je srednji embrij v stadiju 10. Pod vitelinsko membrano se na konicah izboklin apikalne plazmaleme izloča matriks, ki je viden kot tanek rahel sloj, v kasnejših stadijih srednjega embrija pa je matriks sestavljen iz kompaktne elektronsko goste lamine in rahlega materiala pod njo. Nadalje se v poznih stadijih srednjega embrija (14 in 15) pod obstoječim matriksom izloča nov prekutikularni matriks. Elektronsko gosti lamini obeh matriksov sta si strukturno podobni. Sestavljeni sta iz treh tankih slojev, dva

elektronsko gosta sloja in vmesni svetel sloj. Material pod elektronsko gosto lamino je v kasnejšem razvoju bolj kompakten in gost kot pri zgodnejšem matriksu. Embrionalno površino poznega embrija pokriva le en prekutikularni matriks. Za lokalizacijo makromolekul, ki vsebujejo *N*- acetilglukozamin, kar vključuje tudi hitin kot osnovno organsko komponento kutikule, smo na nivoju elektronske mikroskopije (EM) izvedli označevanje z lektini WGA ('wheat germ agglutinin'), konjugiranimi z zlatimi delci, in primerjali sestavo organskega ogrodja prekutikularnih matriksov in kutikule. Prekutikularni matriks ne izkazuje intenzivne vezave lektinov WGA, kar kaže na to, da se sestava organskega ogrodja prekutikularnih matriksov embrijev razlikuje od kutikule odraslih živali. Vezava lektinov na skupke materiala v matriksu poznega embrija v stadiju 18 nakazuje prisotnost glikokonjugatov ali saharidov z *N*- acetilglukozaminom.

Embrionalni razvoj vključuje tudi morfogenezo epidermisa in neprestane spremembe v zunanji morfologiji telesa (rast, razvoj glave in okončin). Prekutikularni matriks, ki je prisoten v določenem obdobju embrionalnega razvoja, mora biti dovolj fleksibilen, da lahko sledi tem spremembam. Strukturne značilnosti prekutikularnega matriksa, kot sta neenakomerna debelina in valovita elektronsko gosta lamina, ki ima večjo površino kot epidermis, kažeta na to, da matriks verjetno lahko sledi rasti embrijev. Prekutikularni matriks se obnavlja v času, ko prihaja do večjih morfoloških sprememb, povezanih z razvojem. Pod vitelinsko membrano smo pri poznem embriju v stadiju 16 opazili skladovnice nagubane elektronsko goste lamine prekutikularnega matriksa, kar kaže na to, da se je prejšnji matriks odluščil z epidermalne površine in da se v času prehoda iz srednjega v pozni embrij prekutikularni matriks zamenja z novim. Prehod iz srednjega v pozni embrij vključuje večje spremembe v zunanji morfologiji embrija, in sicer podaljšanje in ventralni upogib telesa (embrij preide v obliko vejice). Poleg tega se v tem času tudi izleže iz horiona in se okoli svoje vzdolžne osi obrne znotraj vitelinske membrane. Pri tem se pretrga vez z izvenembrionalnim dorzalnim organom (Milatovič in sod., 2010), ki naj bi imel pomembno osmoregulacijsko vlogo v razvoju perakaridnih rakov, kot predlagajo Meschenmoser in sod. (1989). Prekinitev povezave med embrijem in dorzalnim organom je verjetno povezana s spremembami v osmoregulacijski kapaciteti embrija v času prehoda v pozno embriogenezo, kar je lahko dodaten razlog za obnovitev površinskega matriksa. Podobne skladovnice nagubane elektronsko goste lamine kot pri stadiju 16 smo opazili tudi v zadnjem stadiju embriogeneze (stadij 19), kar kaže, da se zadnji prekutikularni matriks odlušči s površine embrija proti koncu embrionalnega razvoja. Takrat se embrij sprosti iz vitelinske membrane in preide v neposreden stik z marzupijsko tekočino.

Prekutikularni matriksi, ki smo jih opisali pri raku enakonožcu *P. scaber*, so ultrastrukturno podobni embrionalnim epidermalnim matriksom, ki so jih opisali pred formacijo kutikule pri drugih členonožcih, med drugimi tudi pri perakaridnih rakih z razvojem v valilniku (Goudeau, 1976; Havemann in sod., 2008). V literaturi obstajajo

različna poimenovanja le-teh, največkrat embrionalna kutikula (Dorn, 1976; Belk, 1987; Konopova in Zrzavy, 2005; Moussian in sod., 2006; Havemann in sod., 2008), lahko pa tudi embrionalna membrana (Ziese in Dorn, 2003) in embrionalna ovojnica (Goudeau, 1976; Goudeau in Lachaise, 1983; Glas in sod., 1997). Kot je znano iz literature, embrionalni epidermis pri različnih vrstah členonožcev izloči različno število apikalnih matriksov, skupno od dva do pet. Med njimi zadnji, ki ga izloči embrij, predstavlja prvo larvalno kutikulo. Vsi prejšnji matriksi se do izleganja embrija razgradijo oziroma odluščijo s površine embrija. O izvoru in funkciji embrionalnih kutikul oziroma prekutikularnih matriksov je v literaturi podanih več razlag. Največkrat predvidevajo, da gre le za ostanek kutikule, ki se je ohranil kot reducirani kutikularni matriks (Morris in Afzelius, 1967; Ziese in Dorn, 2003; Konopova in Zrzavy, 2005; Moussian in sod., 2006). Opazili smo, da je troslojna zgradba elektronsko goste lamine prekutikularnih matriksov podobna zgradbi zunanje epikutikule pri žuželkah, imenovane ovoj ('envelope'), prve strukture nove kutikule, ki se izloči v levitvi (Locke, 2001). O tej podobnosti poročajo tudi v drugih študijah embrionalnih kutikul (Ziese in Dorn, 2003; Konopova in Zrzavy, 2005; Havemann in sod., 2008). Verjetno imajo ti zgodnji epidermalni matriksi med razvojem embrija različne funkcije, kot so zaščita embrija, regulacija prehoda snovi skozi površino embrija in vzdrževanje primernega mikrookolja za formacijo kutikule.

Ultrastrukturne značilnosti epidermalnih celic v embrionalnih stadijih 10 in 13, ko se tvori zgodnji apikalni zunajcelični matriks, kažejo na to, da imajo celice že vzpostavljeno apikalno-bazalno polarnost, kar je povezano tudi z usmerjeno sekrecijo zunajceličnega matriksa. Pri srednjem embriju v stadiju 10 smo opazili še veliko celičnih delitev. Epidermalne celice so mehansko povezane s subapikalnimi adherentnimi stiki. Na mestu stika je pod membranama obeh celic viden sloj gostega materiala, kar kaže, da so že prisotni citoplazemski proteinski kompleksi stika. Manj obsežen in manj elektronsko gost citoplazemski sloj pri nekaterih stikih lahko kaže na to, da ti stiki še niso popolnoma oblikovani. Tepass in Hartenstein (1994) navajata, da je manjša gostota citoplazemskega materiala v stiku opazna pri celicah v delitvi. Razlagata tudi, da se med celično delitvijo medcelični stiki ohranijo, poteka pa izrazito preurejanje citoskeleta, ki je povezan s citoplazemskim materialom stika. Adherentni stiki, opisani tudi med diferenciacijo epitelov pri vinski mušici (Tepass in Hartenstein, 1994; Moussian in sod., 2006), so pomemben element pri vzpostavitvi apikalno-bazalne polarnosti epitelnih celic in ločujejo apikalno in bazolateralno domeno celične membrane (Tepass in sod., 2001; Payre, 2004). Najprej se vzpostavijo točkovni adherentni stiki v lateralnih membranah že v času celularizacije, kasneje pa se ti pomaknejo na apikolateralni rob celic in se povežejo v pas (Tepass in Hartenstein, 1994). Glede na prisotnost subapikalnih adherentnih stikov na vseh subapikalnih celičnih mejah sklepamo, da so verjetno že oblikovani v pas, ki obdaja in povezuje celice. Pri stadiju 13 je epidermis urejen v enojni sloj celic cilindričnih do kubičnih

oblik. Pri embrionalnih stadijih 10 in 13 so v apikalni plazmalemi epidermalnih celic prisotne posamezne daljše cisterne granuliranega endoplazemskega retikuluma (GER), nekaj mitohondrijev, in redkeje tudi posamezne preproste skladovnice Golgijevega aparata, iz česar sklepamo, da imajo celice le manjšo sintetsko aktivnost.

Ultrastruktura epidermalnih celic v razvojnih stadijih 14, 15 in 16, pri katerih nastaja naslednji prekutikularni matriks, kaže na nadaljnjo diferenciacijo in povečano sintetsko ter sekrecijsko aktivnost celic. Epidermis gradijo kubične do ploščate celice. Lateralne plazmaleme so v subapikalnem predelu pogosto nagubane in razvejane, adherentni stiki pa so obsežni. Oblikovanje apikalne plazmaleme v nizke izbokline, dobro razvit GER, nekoliko bolj kompleksen GA in številni z njim povezani vezikli ter elektronsko gosti vezikli pod apikalno plazmalemo kažejo na intenzivnejšo sintetsko in sekrecijsko aktivnost. Pod apikalno celično površino so številni mikrotubuli, ki so v splošnem pomembni pri znotrajceličnem transportu veziklov in pri razporeditvi ter organizaciji Golgijevega aparata in drugih organelov.

3.1.2.2 Diferenciacija kutikule in ultrastruktura epidermisa pri poznih embrijih pred izleganjem in pri marzupijskih mankah

Izraz 'kutikula' smo uporabili za matrikse s poglavitnimi značilnostmi eksoskeletne kutikule rakov, kot so: diferenciacija v značilne horizontalne regije, v epi- in prokutikulo, helikoidalna ureditev hitinsko-proteinskih vlaken, hitinsko ogrodje (vezava lektinov WGA podobno kot pri kutikuli odraslih živali), natančno prileganje apikalni celični membrani, približno enakomerna debelina matriksa vzdolž celice in prisotnost kutikularnih lusk na površini (Neville, 1984; Dillaman in sod., 2013).

Za razliko od zgodnejših razvojnih stadijev embrijev smo pri poznem embriju v stadiju 18 na apikalni plazmalemi epidermalnih celic opazili formacijo matriksa z značilnostmi eksoskeletne kutikule rakov. Kutikula nastaja pod prekutikularnim matriksom in je v tej fazi diferenciacije še zelo tanka. Na površini so že izoblikovane kutikularne luske. V epikutikuli so razvidni posamezni podsloji. Površinski sloji epikutikule so strukturno podobni pet-slojni zunanji epikutikuli odraslega raka enakonožca *P. scaber* (Ziegler, 1997). Pod njimi je elektronsko gost sloj, ki lahko ustreza notranji epikutikuli, a je precej tanjši od notranje epikutikule odraslih živali. Prokutikula strukturno še ni diferencirana v ekso- in endokutikulo in ureditev hitinsko-proteinskih vlaken v podsloje še ni razvidna. Vezava lektinov WGA v liniji novo nastajajoče kutikule kaže na to, da nastaja organsko ogrodje podobno tistemu pri kutikuli odraslih. Epidermalne celice so sploščene, z izrazito nagubano apikalno površino. Pod subapikalno nameščenimi adherentnimi stiki so v medceličnem prostoru prisotne posamezne pregrade (pl. septa). Sklepamo, da gre za začetek tvorbe septiranih medceličnih stikov. Posamezna septa v

medceličnih prostorih epitelov so našli tudi pri poznih embrijih vinske mušice *Drosophila* in jih razložili kot nastajajoče septirane stike (Tepass in Hartenstein, 1994). Ti stiki so pomembni kot paracelularna difuzijska pregrada, sodelovali pa naj bi tudi pri vzpostavitvi in vzdrževanju celične polarnosti, adheziji celic in interakcijah med celicami (Tepass in sod., 2001). Pri vretenčarjih imajo funkcijo paracelularne difuzijske pregrade tesni stiki, ki se nahajajo subapikalno, vendar za razliko od septiranih stikov pri nevretenčarjih, nad adherentnimi stiki. Podobno kot v našem primeru so tudi v študijah epitelne diferenciacije pri vrsti *Drosophila melanogaster* (Tepass in sod., 2001; Moussian, 2006) ugotovili, da se septirani stiki razvojno pojavijo po tem, ko je polarnost epitelnih celic že vzpostavljena. Tepass in sod. (2001) sklepajo, da so ti stiki bolj kot za vzpostavitev pomembni za vzdrževanje celične polarnosti. Iz ultrastrukture epidermalnih celic poznih embrijev v stadiju 18 lahko sklepamo na intenzivno aktivnost GER in predvidevamo, da se še vedno tvori epikutikula, ker je njen notranji sloj veliko tanjši kot kasneje. Prisotne so tudi akumulacije glikogenskih zrnc, o čemer poročajo tudi v drugih študijah epidermalnih celic med sintezo kutikule (Ziegler, 1997; Moussian, 2006).

V nadaljnjem razvoju embrija se eksoskeletna kutikula odebeli in diferencira. V stadiju 19, ko je embrij med ali tik pred izvalitvijo iz vitelinske membrane, so oblikovani vsi glavni kutikularni sloji: epikutikula ter ekso- in endokutikula z razločnimi podsloji. V epidermisu so opazne tudi popolnoma formirane senzile, ultrastrukturno podobne mehanoreceptorskim senzilam pri odraslih živalih *P. scaber* (Ziegler in Altner, 1995). Poleg tega smo opazili, da je kutikula v večini regij odmaknjena od epidermisa in da se ponekod notranji endokutikularni sloji razgrajujejo, kar kaže na obnavljanje kutikule. Epidermalne celice so, podobno kot v stadiju 18, ploščate, vendar njihova površina ni tako izrazito nagubana. Medcelični stiki so dobro diferencirani. Pod adherentnimi stiki pogosto opazimo daljše septirane stike. Citoplazma epidermalnih celic je v embrionalnem stadiju 19 precej redka. Številčnost, velikost in izrazite kriste mitohondrijev nakazujejo intenzivno presnovno aktivnost celic. V primerjavi z epidermalnimi celicami pri srednjih embrijih v stadijih 14 in 15 ter pri poznih embrijih v stadijih 16 in 18, Golgijev aparat in GER nista tako obsežna, manj pa je tudi veziklov. Prisotnost heterogenih vezikularnih struktur v apikalni citoplazmi, ki so podobne multivezikularnim telesom, bi lahko kazala na endocitotsko aktivnost celice.

Po izleganju iz vitelinske membrane se pri ličinki manki tvori nova eksoskeletna kutikula. Morfološke znake razgradnje prve kutikule smo opazili v zadnjem stadiju embriogeneze (stadij 19) in pri eni novo izleženi ličinki, pri kateri smo nad novo kutikulo razločili ostanke prejšnje kutikule. Pred izleganjem je embrij močno ukrivljen znotraj vitelinske membrane, po izleganju pa se telo in okončine sprostijo. V tem času zaradi odsotnosti jajčnih ovojnic pride tudi do direktne izpostavitve ličinke manke marzupijski tekočini. Te spremembe so lahko povezane z menjavo eksoskeletne

kutikule v času prehoda embrija v stadij ličinke. V študijah drugih členonožcev predvidevajo, da je eksoskeletna kutikula, ki nastane med embrionalnim razvojem, ohranjena tudi v prvem stadiju ličinke (Dorn, 1976; Goudeau, 1976; Ziese in Dorn, 2003; Konopova in Zrzavy, 2005; Moussian in sod., 2006; Havemann in sod., 2008), vendar te študije zajemajo proučevanje ultrastrukture kutikule pri embrijih do izleganja iz jajčnih ovojnic. Ultrastrukturne študije kutikule ličink proučujejo stadije ličink v kasnejših levitvah (Locke, 1961, 2001; Anger, 1983), medtem ko prehodno obdobje od izleganja embrija do novo izležene ličinke ultrastrukturno še ni bilo proučeno. Naši rezultati kažejo na to, da prva levitev poteče na začetku razvoja ličinke. To je v skladu s študijami *in vivo* opazovanj intaktnih embrijev in ličink rakov, kjer poročajo o levitvi prve ličinke zelo kmalu po izleganju iz jajčnih ovojnic, stadij med izleganjem in prvo levitvijo pa imenujejo predličinka (angl. prelarva) (Anger, 1983; Helluy in Beltz, 1991).

Pri novo izleženi marzupijski manki so epidermalne celice pokrite s tanko kutikulo, ki se natančno prilega njihovi nagubani površini. Kutikula je v tem stadiju diferencirana v epi- in prokutikulo. Epikutikula ima tanke podsloje, ki so morfološko podobni podslojem epikutikule pri odraslih živalih. V prokutikuli, ki zavzema večji del kutikule, pri osmiranih vzorcih ne razločimo značilnega vzorca ureditve hitinsko-proteinskih vlaken. V ne-osmiranih vzorcih, pri katerih lahko ureditev vlaken bolje razločimo, vidimo, da je ta podobna ureditvi vlaken v eksokutikuli, torej se najprej izločijo lamele eksokutikule, podobno kot pri levitvah odraslih. Izrazitejša vezava lektinov na kutikulo kaže, da je v tem razvojnem stadiju ogrodje kutikule po sestavi že bolj podobno ogrodju kutikule odraslih živali. V tem stadiju opazimo tesno povezanost kutikularnega matriksa s celicami, saj v prokutikulo segajo številni široki citoplazemski izrastki.

V nadaljnjem razvoju marzupijske manke se prokutikula odebeli in je jasno diferencirana v ekso- in endokutikulo, ki sta strukturno zelo podobni ekso- in endokutikuli odraslih živalih. Sklepamo, da je organsko ogrodje kutikule v tem obdobju larvalnega razvoja zelo podobno kot pri kutikuli odraslih, saj je vezava lektinov WGA na kutikulo podobno intenzivna kot v kutikuli odraslih. Citoplazemski izrastki epidermalnih celic, ki segajo v kutikulo, in izbokline apikalne plazmaleme v nekaterih vzorcih kažejo na to, da se kutikularni matriks še tvori.

Značilna ureditev hitinsko-proteinskih vlaken v ekso- in endokutikuli je izrazita v kutikuli poznih marzupijskih mank. V tem razvojnem stadiju prepoznamo tudi za rake enakonožce značilen distalni podsloj eksokutikule, ki vsebuje elektronsko gostejša vlakna. V poznem larvalnem razvoju v valilniku smo opazili tudi odmik kutikule od epidermisa in nastanek levitvenega prostora ter začetne znake razgradnje notranjih endokutikularnih podslojev. Pri večih vzorcih poteka tudi že tvorba nove kutikule. Pozna marzupijska manka torej preide v fazo predlevitve. Epidermalne celice kubične do ploščate oblike. Apikalna celična površina in tanka nova kutikula nad njo sta izrazito

nagubani, podobno kot ob formaciji prve kutikule (embrionalni stadij 18). Pod kratkimi adherentnimi stiki v subapikalnem predelu epidermisa so prisotni septirani stiki, ki zavzemajo precejšen del lateralnih membran in so precej daljši v primerjavi s septiranimi stiki pri embriju v stadiju 19. Apikalna citoplazma je zapolnjena s celičnimi organeli, med njimi obsežen GER, dobro razvit GA in številni z njim povezani vezikli, elektronsko gosti vezikli pod apikalno plazmalemo in številni mitohondriji. Sklepamo na izrazito sintetsko aktivnost epidermalnih celic v tem razvojnem stadiju, kar povezujemo s nastajanjem nove kutikule. Prisotnost plakov na apikalni plazmalemi in vlakna v notranjih delih nove kutikule, kažejo na nalaganje hitina.

3.1.2.3 Ultrastrukturne značilnosti epidermalnih celic med diferenciacijo in v povezavi s sintezo in izločanjem zunajceličnih matriksov

Ultrastruktura epidermalnih celic se med razvojem spreminja v povezavi z diferenciacijo epidermisa in sintezo apikalnih zunajceličnih matriksov. Nekatere značilnosti, ki smo jim sledili v zaporednih razvojnih fazah, interpretiramo kot pokazatelje diferenciacije epidermisa med razvojem. To sta oblikovanje medceličnih stikov in oblika epidermalnih celic, ki se med srednjim in poznim embrionalnim razvojem spreminja od cilindrične preko kubične do ploščate. Preoblikovanje apikalne plazmaleme epidermalnih celic v izbokline z elektronsko gostimi konicami in v daljše citoplazemske izrastke, ki segajo v nastajajoči kutikularni matriks, se pojavlja v zvezi s sintezo in izločanjem apikalnega zunajceličnega matriksa. Nekaterih sprememb ultrastrukturnih značilnosti epidermisa med razvojem pa glede na naše rezultate ne moremo pripisati le enemu procesu. Obsežnost Golgijevega aparata in organizacija ter razporeditev diktiosomov so v različnih razvojnih stadijih različne in se spreminjajo verjetno tako glede na diferenciacijo celice kot tudi glede na fazo sinteze apikalnega matriksa.

Oblikovanje subapikalnih medceličnih stikov je znak diferenciacije apikalno-bazalno polariziranih epitelnih celic. V stadiju srednjega embrija, pri katerem smo opazili prve znake usmerjenega izločanja apikalnega zunajceličnega matriksa, se epidermalne celice še delijo in so mehansko povezane samo s subapikalnimi adherentnimi stiki. Subapikalne adherentne stike so opisali tudi v študijah epitelne diferenciacije pri vinski mušici in so pomembni pri mehanski povezavi celic in pri vzpostavitvi polarnosti epitela (Tepass in Hartenstein, 1994; Tepass in sod., 2001; Payre, 2004). Pri poznem embriju pred izleganjem, ko se tvori prva kutikula, se oblikujejo septirani stiki. Pri embriju v stadiju 18 so prisotna posamezna septa v medceličnem prostoru, ki jih interpretiramo kot nastajajoči septirani stiki. Pri naslednjem razvojnem stadiju (embrij v stadiju 19) pa so bila razvidna daljša neprekinjena zaporedja sept. Pri poznih marzupijskih mankah se septirani stiki podaljšajo in zavzemajo precejšen del lateralnih

membran. Septirani stiki sodelujejo pri vzpostavitvi in vzdrževanju celične polarnosti in so pomembni kot paracelularna difuzijska pregrada (Tepass in sod., 2001).

Apikalna plazmalema epidermalnih celic se med tvorbo apikalnega zunajceličnega matriksa preoblikuje v izbokline z elektronsko gostimi konicami. Nizke izbokline apikalne plazmaleme smo opazili pri epidermalnih celicah v vseh embrionalnih in larvalnih stadijih, ki jih pokriva prekutikularni matriks ali nastajajoča kutikula. Povezujemo jih s sintezo in izločanjem komponent matriksa, saj o podobnih strukturah epitelnih celic med aktivno sintezo kutikule poročajo tudi v drugih študijah. Pogosto so daljše in jih imenujejo mikrovilom podobne strukture. Opisane so pri embrijih in ličinkah žuželk (Locke, 1961; Locke in Huie, 1979; Locke, 2001; Moussian in sod., 2006) ter pri odraslih rakih v levitvi (Koulish in Klepal, 1981; Compere, 1995; Ziegler, 1997; Elliot in Dillaman, 1999; Havemann in sod., 2008; Vittori, 2012). Predvidevamo, da v naši študiji opisane elektronsko goste konice izboklin apikalne plazmaleme ustrezajo tako imenovanim plakom plazmaleme, za katere se predvideva, da so mesta agregacij encimov, kjer poteka organizacija nastajajočih komponent matriksa (Locke in Huie, 1979; Locke, 2001; Moussian, 2010) in ki so jih opisali tudi pri odraslih rakih v levitvi (Koulish in Klepal, 1981; Compere, 1995; Ziegler, 1997). Pri sintezi nove kutikule marzupijskih mank smo opazili tudi drugačne modifikacije apikalne plazmaleme, široke citoplazemske izrastke, ki segajo v kutikulo. Citoplazemski izrastki znotraj pornih kanalov v kutikuli so poznane strukture pri ličinkah žuželk in odraslih rakih med kasnejšo fazo sinteze kutikularnega matriksa. Povezujejo jih z dostavo in izločanjem različnih kutikularnih komponent, med drugim lipidov, tudi do zunanjih delov kutikule (Locke, 1961; Compere in Goffinet, 1987b; Compere, 1991). Ugotovili smo tudi, da je površina epidermalnih celic v tistih razvojnih stadijih, pri katerih se izloča nova kutikula, izrazito nagubana, nova kutikula pa natančno sledi tej liniji nagubanja. To smo opazili v stadijih poznega embrija v stadiju 18, novo izležene marzupijske manke in pozne marzupijske manke. Nagubanje celične površine je v teh stadijih pričakovan pojav, saj tako nastaja nagubana kutikula, ki ima večjo površino kot stara kutikula, kar zagotavlja nadaljnjo rast embrijev in mank.

Spreminjanje organizacije in razporeditve Golgijevega kompleksa med diferenciacijo celic je poznano iz študij nekaterih celic pri sesalcih (Yadav in Linstedt, 2011), vključno z urotelijskimi celicami (Hudoklin in sod., 2009; Erdani Kreft in Višnjar, 2013) in iz študij ličink in imaginalnih diskov pri žuželkah (Zeng in Locke, 1993; Kondylis in sod., 2001; Locke, 2003). Hudoklin in sod. (2009) so ugotovili, da je organizacija in razporeditev Golgijevega aparata (GA) odvisna od stopnje diferenciacije urotelijskih celic. Tako je osnovni GA sestavljen iz nekaj manjših skladovnic kratkih cistern ob jedru. Prehodni GA vsebuje več skladovnic, ki imajo večje in številčnejše cisterne. Kompleksni GA vsebuje mnogo skladovnic, ki so razporejene po večjem delu citoplazme in vsebujejo številne dolge cisterne. Prerazporeditve GA v urotelijskih

celicah se ujemajo z začetkom sinteze t.i. urotelijskih plakov, kar kaže na to, da ima razvoj GA vlogo v diferenciaciji urotelijskih celic v splošnem, predvsem pa pri formaciji urotelijskih plakov (Hudoklin in sod., 2009). Popolnoma razvit Golgijev kompleks je pri žuželkah strukturno preprostejši kot pri vretenčarjih. Skladovnice so posamezne in niso povezane med sabo, kot je to značilno za sesalce, ampak so razporejene po citoplazmi. Pri ličinkah žuželk so ugotovili, da nova skladovnica GA začne nastajati iz veziklov, ki se odcepljajo iz granuliranega endoplazemskega retikuluma. Ti se zlijejo najprej v *cis* cisterne, ki kasneje dozorijo v manjšo skladovnico cistern in nato v popolnoma razvit GA (Locke, 2003). V študiji diferenciacije epitelnih celic v imaginalnih diskih pri ličinkah žuželke *Drosophila melanogaster* so ugotovili, da se morfologija GA jasno spreminja v povezavi z razvojem celic in potrebo po sintezi in izločanju snovi (Kondylis in sod., 2001). Na začetku razvoja diskov so našli majhne skupke veziklov in kratkih tubulov v bližini ER cistern. Iz teh skupkov postopoma nastanejo večji skupki veziklov in daljših tubulov ter posameznih elementov cistern. V bubi, ko so diski že formirani, pa so prisotne skladovnice cistern, okrog cistern pa večje tubularne mreže. Cisterne in skladovnice cistern torej pri žuželkah nastanejo iz veziklov in tubulov, kar so dokazali tudi z lokalizacijo različnih za GA značilnih proteinov v začetnih skupkih veziklov in tubulov (Kondylis in sod., 2001). V naši študiji smo posamezne preproste skladovnice Golgijevega kompleksa našli že v epidermalnih celicah pri srednjem embriju v stadiju 10, kjer so opazni zgodnji znaki izločanja apikalnega matriksa. V embrionalnih stadijih 14 in 15, ko se izloča prekutikularni matriks, je Golgijev aparat nekoliko bolj kompleksen, skladovnice so nekoliko bolj obsežne, nekatere cisterne pa vsebujejo elektronsko gost material. Glede na to, da je Golgijev aparat mesto formiranja in razvrščanja sintetiziranih proteinov na določene lokacije, predvidevamo, da ima v zgodnjih stadijih pomembno vlogo pri usmerjenem izločanju komponent prekutikularnega matriksa. Pri analiziranih osebkih poznega embrija pred izleganjem (stadija 18 in 19) in novo izleženih marzupijskih mank Golgijev aparat v apikalnem delu celice ni bil izrazit. Tem osebkom je skupno to, da nova kutikula ni bila vidna ali pa je bila zelo tanka, torej v zelo zgodnjih fazah tvorbe. Pri poznih marzupijskih mankah, pri katerih poteka sinteza nove kutikule, je bil v apikalni citoplazmi prisoten izrazit Golgijev aparat. Debelina te nove kutikule je primerljiva s prejšnimi vzorci. Vsebina nekaterih cistern Golgijevega aparata je elektronsko gosta, iz njih pa se odcepljajo elektronsko gosti vezikli. Za spremljanje preoblikovanja in prerazporeditve Golgijevega aparata v epidermalnih celicah med razvojem mokric bi bila potrebna natančnejša lokalizacija Golgijevega aparata na večjem številu vzorcev in tudi na nivoju svetlobne mikroskopije.

Naša opažanja kažejo, da so tudi epitelne celice, ki se delijo, pokrite z apikalnim zunajceličnim matriksom. Pri srednjem embriju v stadiju 10 smo opazili, da epidermalna celica v mitozi vsebuje preproste skladovnice Golgijevega aparata, apikalna plazmalema pa je diferencirana v izbokline, na vrhu katerih so razvidne fibrile

zunajceličnega materiala. Za Golgijev aparat sesalčjih celic je značilno, da je med celično delitvijo preoblikovan v močno fragmentirano obliko, Golgijeve membrane pa so razporejene po celotni celici, ki se deli (Yadav in Linstedt, 2011). Za razliko od tega pa v študiji ličink žuželk v levitvi navajajo, da se Golgijev kompleks v epidermalnih celicah med mitozo ne razdeli v vezikle, ampak ostane strukturno in funkcionalno nespremenjen glede na celico v interfazi celičnega cikla. Prav tako ostanejo funkcionalne tudi mikrovilom podobne izbokline apikalne plazmaleme in se lahko izločanje matriksa nemoteno nadaljuje tudi med delitvijo epidermalnih celic (Zeng in Locke, 1993).

Glede na podatke iz literature naj bi bila endocitoza predvidoma povezana s kontrolo sestave kutikule med odlaganjem, resorpcijo stare kutikule, kontrolo sestave levitvene tekočine in recikliranjem membrane iz plazmaleme (Goudeau, 1976; Koulish in Klepal, 1981; Ziegler, 1997; Locke, 2001, 2003). Eden od znakov endocitotske aktivnosti celic je prisotnost multivezikularnih teles v citoplazmi. Ti nastanejo iz endocitotskih veziklov plazmaleme in primarnih lizosomov iz Golgijevega kompleksa. Med levitvijo žuželčjih ličink prisotnost multivezikularnih teles v epidermalnih celicah povezujejo predvsem z dinamično kontrolo kutikularnega okolja med formacijo kutikule, ko potekata tako izločanje kot tudi privzemanje snovi (Locke, 2003). Poleg tega pa naj bi ob koncu tvorbe kutikule potekali endocitoza plakov apikalne plazmaleme in njihova razgradnja v multivezikularnih telesih (Locke in Huie, 1979). V naši študiji smo v apikalni citoplazmi epidermalnih celic pri embriju pred izleganjem v stadiju 19 opazili obsežnejše heterogene strukture v apikalni citoplazmi, ki so podobne multivezikularnim telesom. Predvidevamo, da bi endocitotska aktivnost epidermalnih celic med razvojem znotraj valilnika lahko bila povezana tudi s privzemom snovi iz marzupijske tekočine.

3.1.3 Primerjava diferenciacije eksoskeletne kutikule z diferenciacijo kutikularnega matriksa v črevesu med razvojem mokrice *P. scaber* v valilniku

Epitel prebavne cevi kopenskih vrst rakov enakonožcev, vključno z vrsto *P. scaber*, je ektodermalnega izvora, kar je ena od ključnih prilagoditev te skupine na kopensko življenje (Štrus in sod., 1995). Epitel je enoslojen, celice pa na apikalno površino izločajo kutikularni matriks, sestavljen iz zunanje elektronsko goste epikutikule in notranje elektronsko svetlejše prokutikule. Med razvojem se sprednje in zadnje črevo uvihata iz površinskega ektoderma v obdobju srednjega embrija in se v nadaljnjem razvoju podaljšujeta drug proti drugemu. V enotno cev se povežeta ob prehodu embrija iz srednje v pozno obdobje embriogeneze (Milatovič in sod., 2010). V poznem embriju potekata diferenciacija posameznih delov prebavne cevi in tvorba specializiranih struktur, pri marzupijski ličinki pa je morfološko že diferencirano in pripravljeno na

prehranjevalno vlogo (Štrus in sod., 2008). Diferenciacija kutikularnega matriksa v črevesu med razvojem še ni bila podrobno raziskana.

Zadnje črevo morfološko in funkcionalno delimo na tri osnovne dele: anteriorno komoro, papilatno regijo in rektum. V anteriorni komori je prokutikula nekajkrat debelejša od epikutikule, v njej pa so vidne lamele hitinsko-proteinskih vlaken, v papilatni regiji pa je epikutikula približno enake debeline kot prokutikula in približno trikrat debelejša v primerjavi z epikutikulo v anteriorni komori, lamele hitinsko-proteinskih vlaken pa niso opazne (Mrak in sod., 2013; Bogataj, 2014). Črevesna kutikula ima skupaj s črevesnim epitelom transportno funkcijo v povezavi s transportom vode in sestavin prebavljene hrane (Hryniewiecka-Szyfter in Storch, 1986). V anteriorni komori poteka predvsem prebava hrane in delno tudi absorpcija hranilnih snovi, v papilatni regiji pa potekata predvsem transport ionov in resorpcija vode (Hames in Hopkin, 1989).

V nalogi smo v nekaterih stadijih embrijev in marzupijskih mank analizirali apikalni zunajcelični matriks črevesnih celic in rezultate primerjali z ultrastrukturo epidermalnih matriksov med razvojem raka *P. scaber*. Ugotovili smo, da epitel zadnjega črevesa poznih embrijev v stadijih 16 in 18 apikalno prekriva zunajcelični matriks z izrazito nagubano površino, strukturno podoben prekutikularnemu matriksu, ki v teh stadijih pokriva epidermalne celice. Sestavljen je iz elektronsko goste lamine in elektronsko svetlega materiala pod njo. Podobno kot na površini epidermisa, je v stadiju 18 tudi nad apikalno plazmalemo črevesnih celic razviden novo nastajajoči matriks, ki se prilega apikalni površini celic. Za razliko od matriksa nad epidermisom, smo opazili, da je tanek elektronsko gost sloj novega matriksa v črevesu ponekod še prekinjen. Kutikularne površinske strukture, značilne za črevesno kutikulo, v tem stadiju še niso prisotne. Apikalna plazmalema črevesnih celic je oblikovana v kratke izbokline z elektronsko gostimi konicami, podobno kot je to značilno za epidermalne celice. Struktura apikalnega matriksa črevesnih celic v stadiju 19, ko je embrij blizu izleganja iz vitelinske membrane, že nakazuje podobnost tega matriksa s črevesno kutikulo odraslih. Sestavlja ga troslojna distalna lamina, elektronsko gosta plast pod njo in notranji debel elektronsko svetel sloj. Vendar za razliko od eksoskeleta, kjer so v tem stadiju vsi kutikularni sloji že strukturirani tako kot v kutikuli odraslih, v črevesu diferenciacija slojev še ni izrazita.

Pri zgodnji marzupijski manki črevo pokriva kutikula iz homogene elektronsko svetle prokutikule in nad njo ležeče tanke elektronsko goste epikutikule s tankim površinskim troslojem. V primerjavi s črevesno kutikulo odraslih je kutikula marzupijskih mank znatno tanjša, predvsem pa je manj obsežna elektronsko gosta plast epikutikularnega sloja. Arhitektura kutikule in razmerje debelin epikutikule in prokutikule se v različnih predelih zadnjega črevesa še ne razlikuje, kot je to očitno pri odraslih živalih.

Elektronsko gosta plast epikutikule pod površinskim troslojem je pri poznih marzupijskih mankah nekoliko debelejša, vendar še vedno znatno tanjša kot pri odraslih živalih. Za razliko od prejšnjih stadijev, so pri pozni marzupijski manki prisotni diferencirani kutikularni trni na površini, struktura kutikule v različnih regijah zadnjega črevesa pa je še vedno podobna. Podobno kot pri eksoskeletu so v tem stadiju tudi v črevesu opazni znaki levitve kutikule, torej se črevesna kutikula, tako kot eksoskeletna kutikula, obnovi verjetno kmalu po sprostitvi mank iz valilnika.

V zgodnjih fazah tvorbe kutikule med razvojem se diferenciacija črevesne kutikule in diferenciacija eksoskeletne kutikule ne razlikujeta bistveno, saj gre v obeh primerih za sintezo apikalnega hitinskega matriksa na novo. Specializirane funkcije obeh kutikul v teh fazah verjetno še niso razvite tako kot pri odraslih. Večje strukturne razlike obeh kutikul se pojavijo v poznejših razvojnih stadijih, ko eksoskelet postopno prevzama oporno in zaščitno vlogo, črevesna kutikula pa vlogo pri predelavi, transportu in absorpciji hrane. Vendar pa kutikula v različnih predelih zadnjega črevesa še ni strukturno različna, kot je to značilno za črevesno kutikulo odraslih živali. Prav tako črevesna kutikula pri marzupijskih mankah še ni strukturno tako podobna črevesni kutikuli odraslih živali, kot je to značilno za eksoskeletno kutikulo marzupijskih mank. To morda nakazuje manjši časovni zamik v diferenciaciji črevesne kutikule glede na eksoskeletno kutikulo, vendar bi bile za to trditev potrebne nadaljnje raziskave.

3.1.4 Strukturne povezave med eksoskeletom in mišičnimi celicami med razvojem mokrice *P. scaber*

Za gibanje in stabilnost živali je nujna učinkovita povezava med skeletom in mišicami. Pomemben del mišičnoskeletnega sistema pri členonožcih so povezovalni kompleksi med eksoskeletno kutikulo, specializiranimi epidermalnimi celicami – tenociti in mišičnimi celicami (Bitsch in Bitsch, 2002). Te mehanske povezave vključujejo povezave kompleksnega kutikularnega matriksa z apikalno plazmalemo tenocita in obsežne medcelične povezave med bazalno plazmalemo tenocita in mišično celico. Tako je mišična celica mehansko povezana z epidermisom in kutikulo preko omrežja, ki ga tvorijo citoskeletni elementi, medcelični stiki in povezave celice z matriksom. Podatki o ultrastrukturi in diferenciaciji teh specializiranih mehanskih povezav izvirajo predvsem iz študij vinske mušice (Volk, 1999; Schweitzer in sod., 2010), za druge členonožce pa je podatkov zelo malo. Organizacija strukturnih povezav med eksoskeletom in epitelnimi ter mišičnimi celicami v fazah formacije nove kutikule pri rakih je poznana pretežno iz nekaterih raziskav odraslih živali (Buchholz in Buchholz, 1989; Yamada in Keyser, 2009). Ultrastrukturno smo jih proučili pri raku enakonožcu *P. scaber* pri poznih embrijih pred izleganjem (stadij 19), ko je eksoskeletna kutikula diferencirana v vse sloje in že nekoliko odmaknjena od epidermisa, ter pri poznih

marzupijskih mankah v fazi predlevitve. Pokazali smo, da je pri teh razvojnih stadijih eksoskeletna kutikula že intenzivno mehansko povezana s tenociti in spodaj ležečimi mišicami. Med odmaknjeno kutikulo in apikalno plazmalemo tenocita so številna navpično potekajoča povezovalna vlakna. Tenociti so ultrastrukturno že podobni celicam pri odraslih in vsebujejo apikalno-bazalno usmerjene snope mikrotubulov, ki se nahajajo tudi v neposredni bližini elektronsko gostih plakov pod apikalno oziroma bazalno plazmalemo. Pokazali smo tudi, da je stik med tenocitom in mišično celico pri embriju v stadiju 19 in pri marzupijski manki v osnovi že podoben strukturi pritrditve mišice na eksoskelet odraslih živalih. Ultrastruktura kaže, da je ta stik pri marzupijski manki že izoblikovan. Predvidevamo, da stik med tenocitom in mišično celico pri embriju pred izleganjem še ni popolnoma diferenciran. Pri embriju je namreč sloj materiala pod bazalno membrano tenocita opazno svetlejši in tanjši v primerjavi s slojem, povezanim s plazmalemo mišične celice, medtem ko sta citoplazemska plaka na mestu stika pri odraslih živalih podobne debeline in gostote v obeh celicah. Podobno stanje so opazili tudi pri vinski mušici v študiji *in vitro* primarnih embrionalnih celičnih kultur, kjer poročajo, da je med diferenciacijo znotrajcelični sloj stika v tenocitu tanjši kot v povezani mišični celici (Tucker in sod., 2004).

V raziskavi smo pokazali, da apikalna plazmalema tenocita ostane povezana s kutikularnim matriksom tudi med menjavo eksoskeleta tako pri odraslih kot pri marzupijskih mankah v fazi predlevitve. Na mestih mišičnih pritrditev je namreč stari eksoskelet povezan s spodaj ležečim epitelom preko številnih navpično razporejenih vlaken, ki potekajo od tenocita skozi novo kutikulo in levitveni prostor ter vse do distalnih slojev odmaknjene kutikule. O podobnih rezultatih, ki kažejo na ohranjanje povezav tenocitov z odmaknjenim eksoskeletom v fazi predlevitve, poročajo tudi pri nekaterih odraslih vodnih rakih (Buchholz in Buchholz, 1989; Yamada in Keyser, 2009). Yamada in Keyser (2009) predvidevata, da se ta vlakna podaljšajo pred začetkom nalaganja nove kutikule in da se okrog njih začne nalagati nov epikutikularni material. To kaže, da med menjavo eksoskeleta bolj verjetno poteka reorganizacija vlaken in njihovih povezav s kutikulo, kot pa obširni nastanek novih vlaken. Navpično potekajoča vlakna med kutikulo in tenociti so po vsej verjetnosti končna pritrjevalna mesta pred levitvijo in skupaj z mikrotubuli v tenocitih in s stiki med tenociti in mišičnimi celicami omogočajo vsaj osnovno gibanje živali med to fazo in med samo menjavo eksoskeleta ter ohranjajo integriteto integumenta med levitvijo.

Iz teh rezultatov sklepamo, da je pri zadnjem embrionalnem stadiju in pri marzupijskih mankah strukturno ogrodje mišičnoskeletnih povezav že osnovano in omogoča gibanje živali znotraj valilnika. To je v skladu z našim opazovanjem marzupijskih mank, ki so znotraj valilnika zmožne gibanja telesa in okončin, in s študijo Milatovič in sod. (2010), v kateri poročajo o aktivnem premikanju embrija med izleganjem iz vitelinske membrane.

3.1.5 Kalcifikacija eksoskeletne kutikule marzupijskih mank mokrice *P. scaber*

Mineralna sestava eksoskeletne kutikule rakov je precej dobro raziskana pri odraslih rakih, tudi pri rakih enakonožcih. Znano je, da eksoskeletna kutikula vsebuje kalcijev karbonat v kristalni obliki (kalcit in magnezijev kalcit), amorfni kalcijev karbonat (ACC) in amorfni kalcijev fosfat (ACP) (Roer in Dillaman, 1984; Becker in sod., 2005; Dillaman in sod., 2005; Hild in sod., 2008, 2009; Neues in sod., 2011; Seidl in sod., 2011; Luquet, 2012). Kalcifikacija hitinskih matriksov med embrionalnim in larvalnim razvojem rakov še ni poznana. V nalogi smo želeli ugotoviti, ali je kutikula marzupijskih mank raka enakonožca *P. scaber* že kalcificirana. Kot osnovo za nadaljnje zahtevnejše analize kalcija v tkivu smo uporabili histokemijsko lokalizacijo kalcificiranega tkiva z barvilom alizarin rdeče S (ARS). Ta metoda je hitra, enostavna in poceni, kar nam omogoča pregled velikega števila vzorcev. Da bi to metodo, ki se sicer uporablja pretežno v histologiji vretenčarjev, optimizirali za hitinske matrikse, smo izvedli več različnih tehnik fiksacije in barvanja. Diferencialno barvanje smo dosegli z naslednjimi postopki metode ARS: (a) fiksacija z nevtralno raztopino formaldehida, ki ji je sledilo barvanje parafinskih rezin z eno izmed raztopin ARS: ARS 1 (pH 9), ARS 2 (pH 6.4) ali ARS 3 (pH 4.8); (b) barvanje zamrznjenih rezin vzorcev, fiksiranih v formaldehidu in barvanih z raztopino ARS 1 (pH 9). Pri teh postopkih se je izrazito diferencialno obarvala tako kutikula odraslih živali, ki smo jo uporabili kot pozitivno kontrolo, kot tudi kutikula marzupijskih mank. Pri negativnih kontrolah s predhodno dekalcifikacijo vzorcev v raztopini EDTA se kutikula odraslih in mank ni obarvala. Rezultati kažejo na znatno kalcifikacijo eksoskeletne kutikule že v larvalnem obdobju pred sprostitvijo iz valilnika v zunanje okolje.

Pri vseh nadaljnjih analiznih metodah, ki smo jih uporabili za elementno analizo (energijsko-disperzijska rentgenska spektrometrija - EDXS) in analizo mineralov (Ramanska spektroskopija), smo vzorce fiksirali v metanolu. Da bi v vzorcih ohranili mineralno fazo, vključno z amorfnimi oblikami mineralov, smo jih v skladu s podatki iz literature fiksirali v metanolu in jih nismo izpostavljali vodnim raztopinam (Brečević in Nielsen, 1989; Becker in sod., 2003). Z EDXS na vrstičnem elektronskem mikroskopu s poljsko emisijo (FE-SEM) smo izvedli natančnejšo detekcijo kalcija v tkivu. EDXS je analizna metoda elektronske mikroskopije, pri kateri z detekcijo rentgenskih žarkov, ki nastanejo pri interakciji elektronskega žarka z atomi v vzorcu, ugotavljamo elementno sestavo tkiva. Elementna sestava kutikule odraslih je poznana iz več študij rakov, vključno z raki enakonožci (Compere in sod., 1992; Becker in sod., 2005; Romano in sod., 2007; Hild sod., 2008, 2009; Seidl in sod., 2011). Glavni elementi odrasle kutikule rakov so ogljik, kisik in kalcij, jasno pa se pokaže tudi prisotnost fosforja, magnezija in žvepla. Večina fosforja v kutikuli je v obliki amorfnega kalcijevega fosfata, kar so pokazali s študijo kombinacije različnih analiznih metod: rentgenske difrakcije (XRD), rentgenske absorpcijske spektroskopije (EXAFS) in atomske absorpcijske

spektroskopije (AAS) (Becker in sod., 2005). Precejšen delež magnezija se nahaja v kristalu kalcita in tvori magnezijev kalcit, ki doda trdnost kutikuli (Becker in sod., 2005). Za referenco nadaljnjim EDXS meritvam kutikule marzupijskih mank smo najprej analizirali elementno sestavo kutikule odrasle mokrice *P. scaber,* kjer smo dobili podobne rezultate kot v omenjenih študijah. Analizirali smo dva zaporedna stadija zgodnjih marzupijskih mank. Naši rezultati EDXS analiz kažejo, da je elementna sestava kutikule pri napredni zgodnji marzupijski manki podobna kot v kutikuli odraslih živali. Kalcijevi vrhovi v spektrih so visoki, izraziti pa so tudi vrhovi magnezija, fosforja, ogljika, kisika in žvepla. Kalcijevi vrhovi so nekajkrat višji od fosforjevih, kar je značilno tudi za kutikulo odraslih živali pri različnih vrstah rakov enakonožcev. Iz tega sklepamo, da je kutikula v tem stadiju že kalcificirana, podobno kot pri odraslih živalih. EDXS analize površine novo izležene zgodnje marzupijske manke kažejo prisotnost kalcija, magnezija in fosforja v kutikuli, značilnih elementov kalcificirane kutikule odraslih rakov. Kalcijev vrh v spektru je pri tem stadiju manke nizek v primerjavi s posnetimi spektri kutikule odraslih živali. Razmerje med kalcijevim in fosforjevim vrhom je pri vseh izmerjenih spektrih manjše od 1, kar je precej manj v primerjavi z značilnim visokim razmerjem vrhov kalcija in fosforja v kutikuli odraslih. Zaključimo lahko, da se pri novo izleženih marzupijskih mankah že akumulira kalcij v kutikuli in da se kutikula po mineralni sestavi razlikuje od kutikule odraslih živali.

Analizo mineralnih oblik kalcija v kutikuli raka enakonožca *P. scaber* smo izvedli z Ramansko spektroskopijo. Uporabili smo Ramanski spektrometer Horiba Jobin-Yvon T64000, vezan na konfokalni fluorescenčni mikroskop z argon-ionskim laserskim virom svetlobe z ekscitacijsko valovno dolžino 514,5 nm. Ramanska spektroskopija je analizna tehnika, ki zazna vibracije molekul v vzorcu na osnovi neelastičnega sipanja monokromatske svetlobe, ki nastane zaradi interakcij s kemijskimi vezmi v molekulah vzorca. S to tehniko pridobimo podatke o kemijski strukturi molekul v vzorcu. Za primerjavo smo najprej izvedli meritve na vzorcih tergitov in sternalih depozitov odraslih živali. V nadaljevanju smo analizirali tri zaporedne stadije marzupijskih mank: novo izležena zgodnja manka, napredna zgodnja manka in srednja manka. Vzorce smo pripravili na tri načine: (i) fiksacija v metanolu, sušenje na zraku in meritve na izpostavljeni površini; (ii) fiksacija v metanolu, vklapljanje v parafinski vosek, rezanje 15-µm debelih rezin, prenos rezin na suho objektno stekelce in meritve na rezinah; (iii) meritve na vzorcih brez predhodnega izpostavljanja kemikalijam ali vodi (pregl. 1). Za vsak Ramanski spekter v območju valovnega števila 800-1400 cm^{-1} smo izvedli 30 meritev po 100 sekund in izračunali povprečje z računalniškim programom LabSpec. Za Ramanske spektre v območju 60-1840 cm^{-1} smo izvedli 20 meritev po 30 sekund in s programom izračunali povprečje. S programom LabSpec smo izvedli tudi odštevanje signalov ozadja in glajenje spektrov.

Rezultati analiz Ramanske spektroskopije na različnih vzorcih so povzeti v preglednici 1. Ramanski spektri, ki so bili izmerjeni v območju valovnega števila 800-1400 cm^{-1}, so prikazani na sliki 19. Za primerjavo smo najprej analizirali notranjo površino tergita odraslih živali v fazi med levitvama. Nato smo izvedli meritve dorzalne površine intaktne napredne zgodnje marzupijske manke. Spekter kutikule pri odraslih kaže izrazit vrh pri 1091 cm^{-1}, značilen za karbonat (Rutt in Nicola, 1974) in prisoten tudi pri Ramanskih spektrih kutikule odraslih rakov enakonožcev v drugih študijah (Hild in sod., 2008, 2009). Izrazit vrh karbonata je razviden tudi pri spektru površine manke. V tem območju spektra ne moremo razlikovati med amorfnim kalcijevim karbonatom (ACC) in kalcitom. Vrh pri 960 cm^{-1} kaže na prisotnost amorfnega kalcijevega fosfata (ACP) (Sauer in sod., 1994), ki je značilen mineral kalcificirane kutikule rakov. Tudi pri spektru površine manke opazimo manjši vrh pri tej vrednosti, ki pa se lahko prekriva z vrhom za C-N vezi v molekulah hitina (957 cm^{-1}). Ker pa smo z EDXS analizami pokazali znatno prisotnost fosforja v kutikuli tega stadija manke, sklepamo, da vrh pri 960 cm^{-1} v Ramanskem spektru kaže tudi na prisotnost ACP. Ostali vrhovi Ramanskih spektrov obeh vzorcev (kutikule odraslih in mank) demonstrirajo različne organske komponente kutikule. Za hitin značilni vrhovi, ki so jih pokazali tudi Hild in sod. (2008) v kutikuli odraslih živali, so pri: 900 in 957 cm^{-1} za C-N vezi; 1045 cm^{-1} za C-O in C-N vezi; 1116 in 1211 cm^{-1} za C-C vezi; vrhovi od 1230 do 1400 cm^{-1} za CH_2.

Meritve Ramanske spektroskopije v širšem območju valovnega števila (60-1840 cm^{-1}) prikazuje slika 20. Za interpretacijo prisotnosti mineralnih komponent v kutikuli marzupijskih mank smo spektre kutikule marzupijskih mank primerjali s spektrom kutikule odraslih in s spektrom sternalnih depozitov. Sternalni depoziti so založne strukture kalcija, ki nastanejo, ko se v fazi predlevitve odraslih živali med epidermisom in staro kutikulo naložijo kalcijevi minerali v obliki ACC (Ziegler, 1994). Pri meritvi v metanolu fiksiranih in posušenih sternalnih depozitov odrasle živali v fazi predlevitve smo dobili referenčni spekter za ACC, kar je v skladu s predhodnimi analizami drugih avtorjev (sl. 20, spekter A). Spekter sprednjega tergita odraslih živali v fazi predlevitve (sl. 20, spekter B) smo izmerili takoj po usmrtitvi živali in brez predhodnega izpostavljanja kemikalijam ali vodi. Meritev smo izvedli na prečnem prelomu tergita. Najvišji vrh v spektrih kutikule odraslih in depozitov (1081 oziroma 1085 cm^{-1}) je značilen za karbonat. V obeh spektrih je prisoten tudi vrh okoli 715 cm^{-1}, značilen za oba polimorfa kalcijevega karbonata (Gierlinger in sod., 2013). Za amorfne faze je značilno, da imajo širše in manj intenzivne vrhove v Ramanskih spektrih kot kristalne faze, kar vidimo tudi v razliki spektrov depozitov in kutikule odraslih. V spektru kutikule odraslih sta prisotna tudi vrhova pri 150 cm^{-1} in 278 cm^{-1}, ki sta značilna za kalcit (Rutt in Nicola, 1974) in nizek vrh pri 960 cm^{-1}, ki je značilen za ACP (Sauer in sod., 1994). Spektri kutikule in notranjih tkiv mank so izmerjeni na parafinskih rezinah. Spekter kutikule srednje marzupijske manke (sl. 20, spekter C) ima podobne vrhove mineralnih komponent kot spekter kutikule odraslih. Vrh pri 1087 cm^{-1} kaže na

prisotnost karbonata, vrhova pri 713 cm^{-1} in 278 cm^{-1} pa na prevladovanje kristalne oblike kalcijevega karbonata (kalcita). Nizek, skoraj neznaten vrh pri 960 cm^{-1}, kaže na prisotnost manjše količine ACP v kutikuli srednje marzupijske manke. V primerjavi s tem, spekter notranjega tkiva pri isti manki (sl. 20, spekter Č) ne vsebuje vrhov, ki so značilni za mineralne komponente kutikule. Pri kutikuli novo izležene zgodnje marzupijske manke (sl. 20, spekter D) kalcijevi minerali v mejah detekcije Ramanske spektroskopije niso zaznani. Ostali vrhovi predvidoma pripadajo organskim komponentam kutikule ter okoliškega tkiva.

Preglednica 1: Pregled analiz mineralnih oblik kalcija z Ramansko spektroskopijo različnih vzorcev kutikule raka enakonožca *P. scaber*

Table 1: Overview of calcium mineral forms analyses by Raman spectroscopy of different *P. scaber* cuticle samples

VZOREC	PRIPRAVA VZORCA	OBMOČJE MERITEV (valovno število)	REZULTATI vrhovi v Ramanskih spektrih (cm^{-1})	
Kutikula odrasle živali v fazi med levitvama - notranja površina tergita	fiksacija v metanolu in sušenje na zraku	800-1400 cm^{-1}	1091 cm^{-1} (izrazit) karbonat	960 cm^{-1} ACP
Kutikula odrasle živali v predlevitvi - sprednji tergit	izolacija (brez fiksacije) in prečni prelom tergita	60-1840 cm^{-1}	1085 cm^{-1} (izrazit, ozek) 715 cm^{-1} 150 cm^{-1} in 278 cm^{-1} kalcit možno tudi ACC	960 cm^{-1} ACP
Sternalni depoziti odraslih živali	fiksacija v metanolu in sušenje na zraku	60-1840 cm^{-1}	1081 cm^{-1} (izrazit, širok) 718 cm^{-1} ACC	/
Kutikula srednje marzupijske manke	fiksacija v metanolu in priprava parafinskih rezin	60-1840 cm^{-1}	1087 cm^{-1} (izrazit, ozek) 713 cm^{-1} 278 cm^{-1} kalcit možno tudi ACC	960 cm^{-1} (neizrazito) ACP
Napredna zgodnja marzupijska manka - dorzalna površina	fiksacija v metanolu in sušenje na zraku	800-1400 cm^{-1}	1092 cm^{-1} (izrazit) karbonat	961 cm^{-1} ACP
Kutikula novo izležene zgodnje marzupijske manke	fiksacija v metanolu in priprava parafinskih rezin	60-1840 cm^{-1}	/	/
Notranje tkivo srednje marzupijske manke	fiksacija v metanolu in priprava parafinskih rezin	60-1840 cm^{-1}	/	/

ACC: amorfni kalcijev karbonat
ACP: amorfni kalcijev fosfat
/ : vrhovi, značilni za kalcijeve minerale, niso prisotni

Z različnimi metodami pridobljeni rezultati v naši nalogi dokazujejo, da je kutikula marzupijske ličinke manke kalcificirana, o čemer prej ni bilo podatkov. Iz rezultatov Ramanske spektroskopije in EDXS analiz lahko sklepamo, da je mineralne komponente v kutikuli novo izležene zgodnje marzupijske manke še relativno malo. EDXS analize dokazujejo prisotnost kalcija in fosforja v kutikuli novo izležene manke, Ramanska spektroskopija pa mineralov ne zazna. To pomeni, da je količina mineralov pod mejo detekcije Ramanske spektroskopije in predvidevamo, da se nalaganje mineralov začne predvsem v amorfni obliki. Vrhovi mineralnih komponent Ramanskih spektrov kutikule naprednih zgodnjih marzupijskih mank in srednjih marzupijskih mank so bolj izraziti in bolj podobni spektrom kutikule odrasle živali, kar nakazuje na to, da je mineralna sestava kutikule v teh stadijih že podobna mineralni sestavi kutikule odraslih. Kalcifikacija eksoskeleta služi utrjevanju kutikularnega matriksa. Trdnost eksoskeletne kutikule mank med razvojem v valilniku je pomembna za oporo in gibanje mank v valilniku. Opazili smo naraščujočo mobilnost marzupijskih mank med razvojem, kar omogoča tudi sprostitev iz valilnika in gibanje mank takoj po tem. Kutikula tudi ščiti manke pred potencialnimi fiziološkimi stresi zaradi osmotskih in ionskih sprememb v marzupijskem okolju. Naši rezultati so v skladu s študijo osmotske tolerance embrijev in mank vrste *Armadillidium vulgare*, v kateri so ugotovili, da ima marzupijska manka kljub odsotnosti jajčnih ovojnic široko toleranco na osmotske ekstreme. To lastnost so pripisali domnevni kalcifikaciji kutikule marzupijske manke (Surbida in Wright, 2001). Nadalje so naši rezultati tudi v skladu s študijo Ouyang in Wright (2005), ki sta s tehniko atomske absorpcijske spektroskopije (AAS) merila celotno koncentracijo kalcija v različnih razvojnih stadijih izopoda *A. vulgare* in ugotovila, da se ta znatno poveča v srednjih stadijih razvoja marzupijske manke. Opazili so tudi, da manka med razvojem v valilniku uživa marzupijsko tekočino in predvidevali, da s tem pridobiva kalcij.

3.2 SKLEPI

Jajčni ovojnici embrijev mokrice *P. scaber*, horion in vitelinska membrana, sta tanjši od jajčnih ovojnic jajčec iz ovarija vinske mušice *Drosophila melanogaster*. Struktura zunanje jajčne ovojnice, horiona embrijev mokric je iz enega samega homogenega sloja z majhnimi lakunami in je enostavnejša kot struktura horiona jajčec vinske mušice. Vitelinska membrana je sestavljena iz obsežnega homogenega sloja, nad katerim je tanek elektronsko gost sloj in tanek elektronsko svetel sloj z nagubano površino, in je po strukturi podobna vitelinski membrani vinske mušice. Razlike v zgradbi ovojnic so odraz različnih okoljskih razmer, v katerih poteka embrionalni razvoj mokrice in vinske mušice.

Med ontogenetskim razvojem raka enakonožca *P. scaber* v valilniku, epidermis tvori več različnih apikalnih zunajceličnih matriksov, ki se zamenjajo v odvisnosti od morfoloških sprememb, povezanih z razvojem in rastjo embrija ter ličinke.

Najbolj zgodnja embrionalna faza, pri kateri smo opazili izločanje epidermalnega matriksa nad apikalno plazmalemo, je srednji embrij v stadiju 10. V nadaljnjih stadijih srednjega embrija se zaporedno izločita najmanj dva prekutikularna matriksa, ki se strukturno in po sestavi organskega ogrodja, razlikujeta od eksoskeletne kutikule odraslih rakov. S površine embrija se odluščita med izleganjem embrija iz horiona in med izleganjem iz vitelinske membrane. Ti matriksi imajo verjetno več funkcij, kot so zaščita embrija, regulacija prehoda snovi skozi površino embrija in vzdrževanje primernega mikrookolja za tvorbo kutikule.

Prvi kutikularni matriks epidermisa se tvori v zaključnem obdobju embriogeneze. Kutikula je pri poznem embriju v stadiju 18 tanka in sestavljena iz epikutikule in prokutikule, do izleganja iz vitelinske membrane pa se odebeli in diferencira v epi-, ekso- in endokutikulo s podsloji. Na podlagi znakov apolize pri poznem embriju v stadiju 19 in strukture kutikule pri novo izleženi marzupijski manki predvidevamo, da prva levitev poteče na začetku razvoja ličinke. Iz vitelinske membrane izležena manka ima novo kutikulo, sestavljeno iz epikutikule in prokutikule. Glede na vezavo lektina WGA sklepamo, da je organsko ogrodje te kutikule podobno kot v kutikuli odraslih. Rezultati EDXS analiz kažejo, da se pri tem stadiju manke v kutikuli akumulira kalcij. Razmerje med kalcijevim in fosforjevim vrhom EDXS analiz je manjše od 1, v primerjavi z značilnim visokim razmerjem vrhov kalcija in fosforja pri kutikuli odraslih. Sklepamo, da je količine mineralov v kutikuli novo izležene marzupijske manke malo in so pod mejo detekcije Ramanske spektroskopije, saj jih s to tehniko ne zaznamo. V nadaljnjem razvoju marzupijske manke se poveča debelina kutikule, kutikularni sloji se diferencirajo v epi-, ekso- in endokutikulo, kalcifikacija kutikule je bolj izrazita. Vezava lektinov WGA na kutikulo v tem obdobju larvalnega razvoja je intenzivna, kar kaže, da je organsko ogrodje te kutikule podobno kot v kutikuli odraslih. Rezultati kombinacij različnih analiznih metod kažejo na to, da sta elementna in mineralna sestava kutikule naprednih zgodnjih marzupijskih mank in srednjih marzupijskih mank že podobni sestavi kutikule odraslih živali. Strukturno organizirana in kalcificirana kutikula marzupijskih mank prevzema zaščitno in oporno funkcijo eksoskeleta in omogoča gibanje mank, ki smo ga opazili v valilniku. Pozna marzupijska manka je v fazi predlevitve, kar sklepamo po odmiku kutikule od epidermisa (apoliza), razgrajevanju notranjih kutikularnih slojev in sintezi nove kutikule. To nakazuje, da se levi kmalu po sprostitvi iz marzupija.

Ultrastrukturna organizacija epidermalnih celic se med razvojem spreminja v povezavi z diferenciacijo epidermisa in sintezo apikalnih zunajceličnih matriksov. V skladu z

zaporednimi razvojnimi stadiji embrijev in mank poteka oblikovanje subapikalnih medceličnih stikov, kar je pokazatelj diferenciacije polariziranih epitelnih celic v apikalno-bazalni smeri. V stadiju srednjega embrija, pri katerem smo opazili prvi epidermalni apikalni matriks, so epidermalne celice mehansko povezane s subapikalnimi adherentnimi stiki. Pri poznem embriju pred izleganjem, ko se tvori prva kutikula, se oblikujejo septirani stiki, ki so pomembni kot paracelularna difuzijska pregrada. Epidermalne celice so pri srednjem embriju v stadiju 10 in 13 cilindričnih do kubičnih oblik, v stadijih 14, 15 in 16 kubičnih do ploščatih oblik, pri poznem embriju v stadiju 18 pa so ploščate. V povezavi s sintezo in izločanjem apikalnih zunajceličnih matriksov se apikalna plazmalema epidermalnih celic pri različnih razvojnih stadijih preoblikuje v nizke izbokline z elektronsko gostimi vrhovi. Pri sintezi nove kutikule so prisotni tudi široki citoplazemski izrastki, ki segajo v kutikulo. Obsežnost, organizacija in razporeditev Golgijevega aparata so v različnih razvojnih stadijih različne in se spreminjajo verjetno tako glede na diferenciacijo celice kot tudi glede na fazo sinteze apikalnega matriksa. Posamezne preproste skladovnice Golgijevega kompleksa vsebujejo že epidermalne celice srednjega embrija v stadiju 10, v nadaljnjih stadijih pa je organiziranost Golgijevega aparata zelo raznolika.

Eksoskeletna kutikula je pri poznem embriju pred izleganjem in pri marzupijski manki že intenzivno mehansko povezana s tenociti in spodaj ležečimi mišicami. Med kutikulo in apikalno plazmalemo tenocita so številna navpično potekajoča povezovalna vlakna. Tenociti so ultrastrukturno podobni celicam pri odraslih in vsebujejo snope mikrotubulov, ki potekajo v apikalno-bazalni smeri. Stik med tenocitom in mišično celico je tudi v osnovi že podoben strukturi stika pri odraslih živalih. Povezave tenocita s kutikularnim matriksom so ohranjene tudi med menjavo eksoskeleta, tako pri odraslih kot pri razvojnih stadijih v fazi predlevitve.

Zgodnja faza diferenciacije črevesne kutikule med razvojem ni bistveno drugačna od diferenciacije eksoskeletne kutikule, v obeh primerih se apikalni hitinski matriks sintetizira na novo. Pri poznih embrijih v stadijih 16 in 18 je v črevesu prisoten apikalni zunajcelični matriks, ki je strukturno podoben prekutikularnemu matriksu epidermalnih celic. Pri poznem embriju v stadiju 19 struktura apikalnega matriksa črevesnih celic že kaže podobnost s strukturo črevesne kutikule odraslih živali, vendar diferenciacija slojev še ni izrazita. Črevesna kutikula marzupijskih mank je še bolj podobna črevesni kutikuli odraslih živali, vendar ne v tolikšni meri, kot je to značilno za podobnost eksoskeletne kutikule marzupijskih mank in odraslih. Za razliko od odraslih živali je črevesna kutikula marzupijski mank tanjša, elektronsko gosta plast epikutikule je bistveno manj izrazita, prokutikula anteriorne komore še ni diferencirana v podsloje, v različnih predelih zadnjega črevesa je kutikula pretežno enaka, so pa že oblikovani kutikularni trni. Pri pozni marzupijski manki so, tako kot pri eksoskeletu, tudi v črevesu opazni znaki levitve kutikule.

4 POVZETEK (SUMMARY)

4.1 POVZETEK

Eksoskeletna kutikula rakov je apikalni zunajcelični matriks epidermisa iz hitinskega organskega ogrodja in utrjen s sklerotizacijo in kalcifikacijo. Organsko fazo sestavljajo hitinsko-proteinska vlakna, različni proteini in lipidi, mineralno fazo pa kalcijev karbonat v kristalni obliki (kalcit in Mg-kalcit), amorfni kalcijev karbonat (ACC) in amorfni kalcijev fosfat (ACP). Organizirana je v tri glavne vodoravne sloje: epikutikulo, eksokutikulo in endokutikulo, ki se razlikujejo v strukturi, funkciji in molekulski sestavi. Tvorba nove kutikule poteka med razvojem embrijev in ličink ter ob vsaki levitvi odraslih osebkov. Pomembni procesi pri tvorbi nove kutikule so sinteza in oblikovanje strukture organskega ogrodja, kalcifikacija in oblikovanje povezav z mišicami.

Embriji kopenskih rakov enakonožcev (Isopoda: Oniscidea) se razvijajo v vodnem okolju valilnika (marzupija), vrečasti strukturi na trebušni strani samice. Embriji se izležejo v ličinke manke, ki nadaljujejo razvoj v valilniku še približno teden dni. V različnih stadijih razvoja so embriji obdani z različnimi zaščitnimi matriksi, jajčnimi ovojnicami, ki so prisotne od začetka razvoja, in zunajceličnimi matriksi epidermisa, ki se tvorijo kasneje med razvojem. Pri členonožcih sta prisotni dve jajčni ovojnici, zunanja je horion, notranja ovojnica je vitelinska membrana. Pri rakih enakonožcih osmotsko in ionsko regulacijo embrijskega mikrookolja uravnavajo jajčne ovojnice in dorzalni organ embrija, delno pa jo zagotavlja sestava marzupijske tekočine. Embriji raka enakonožca *Porcellio scaber* se iz horiona izležejo pri prehodu v obdobje poznega embrija, ko se spremeni tudi zunanja oblika embrija, izleganje iz vitelinske membrane pa označuje konec embrionalnega razvoja in začetek razvoja ličinke.

O diferenciaciji zgodnjih hitinskih matriksov med embrionalnim razvojem je največ znanega pri žuželkah, predvsem iz raziskav vinske mušice *Drosophila melanogaster*. Diferenciacija hitinskih matriksov med razvojem rakov je slabo poznana, predvsem pa so redke ultrastrukturne študije. Nekaj histoloških raziskav ter *in vivo* opazovanj intaktnih embrijev in ličink je bilo narejenih pri deseteronožcih (Decapoda), škrgonožcih (Branchiopoda) in postranicah (Amphipoda), nedavna ultrastrukturna študija diferenciacije kutikule pa pri embrijih vodne postranice *Parhyale hawaiensis*. Kot je znano iz študij odraslih rakov med levitvijo, ličink žuželk med levitvijo in embrijev žuželk, se med tvorbo hitinskega matriksa spreminjata oblika in ultrastruktura epitelnih celic. Sinteza komponent kutikule ali njihovih prekurzorjev se začne v citosolu in v organelih polariziranih epitelnih celic, ti pa nato potujejo skozi apikalno plazmalemo v zunajcelični prostor. Tvorba apikalnih epidermalnih matriksov med razvojem v valilniku pri rakih enakonožcih še ni bila podrobno raziskana. O

kalcifikaciji hitinskih matriksov med razvojem ni podatkov. Eksoskelet členonožcev je mehansko povezan z mišicami preko specializiranih povezav kutikularnega matriksa z apikalno plazmalemo specializirane epitelne celice (tenocita) in preko povezav tenocita z mišicami. Pri teh povezavah je udeleženo kompleksno omrežje citoskeletnih elementov, medceličnih stikov in povezav celic z matriksom. O oblikovanju strukturnih povezav med eksoskeletom in mišicami med embrionalnim razvojem rakov je podatkov zelo malo. Kutikularni matriks je prisoten tudi na apikalni površini črevesnih celic, njegova struktura pa je drugačna od strukture eksoskeletne kutikule. Črevesna kutikula je sestavljena iz zunanje elektronsko goste epikutikule in notranje elektronsko svetlejše prokutikule. Diferenciacija kutikularnega matriksa v črevesu med razvojem rakov še ni bila podrobno raziskana.

Glavni namen doktorskega raziskovalnega dela je pojasnitev diferenciacije eksoskeletne kutikule raka enakonožca *P. scaber* med embrionalnim razvojem in razvojem ličink mank v valilniku, s poudarkom na raziskavi funkcionalne ultrastrukture in sestave. Izvedli smo ultrastrukturno karakterizacijo apikalnih zunajceličnih matriksov epidermalnih celic, jajčnih ovojnic in črevesnih apikalnih matriksov. Poleg ultrastrukture smo analizirali tudi sestavo apikalnih epidermalnih matriksov, s poudarkom na lokalizaciji makromolekul, ki vsebujejo *N*- acetilglukozamin, kar vključuje tudi hitin, in analizi elementne in mineralne sestave kutikule. Del raziskav je bil namenjen analizi nekaterih ultrastrukturnih značilnosti epidermalnih celic v povezavi z razvojem in s sintezo apikalnega matriksa ter karakterizaciji strukturnih povezav kutikule s specializiranimi epidermalnimi celicami (tenociti) in mišicami pri embrijih in marzupijskih mankah.

Osrednji objekt v raziskavi je modelni organizem *P. scaber* iz skupine kopenskih rakov enakonožcev. Za identifikacijo posameznih razvojnih stadijev te vrste smo uporabili opisan razvojni sistem (Milatovič in sod., 2010). Razvoj različnih stadijev marzupijskih mank ni bil natančno opisan, zato smo na novo opisali štiri zaporedne stadije marzupijskih mank. Za ultrastrukturne analize na presevnem (TEM) in vrstičnem elektronskem mikroskopu (SEM) smo vzorce fiksirali v 2.5 % glutaraldehidu v 0.1 M kakodilatnem pufru (pH = 7.2) in postfiksirali v 1 % osmijevem tetroksidu. Za presevno EM smo jih vklopili v epoksi smolo in pripravili ultratanke rezine. Za vrstično EM smo embrije kemijsko posušili in naprašili z zlatom. Za lokalizacijo makromolekul, ki vsebujejo *N*- acetilglukozamin, kar vključuje tudi hitin kot osnovno organsko komponento kutikule, smo izvedli označevanje z lektini WGA ('wheat germ agglutinin'), konjugiranimi z zlatimi delci velikosti 10 nm. V ta namen smo vzorce fiksirali v 2 % paraformaldehidu in 0.25 % glutaraldehidu v 0.1 M pufru Hepes (pH = 7.2) in jih nato vklopili v akrilno smolo. Označevali smo ultratanke rezine embrijev in mank, istočasno pa za pozitivno kontrolo še rezine z EDTA dekalcificirane kutikule odraslih. Za ugotavljanje kalcifikacije kutikule smo izvedli različne metode. Najprej

smo kalcificirano tkivo histokemijsko lokalizirali z barvilom alizarin rdeče S (ARS), pri čemer smo na marzupijskih mankah in kutikulah odraslih živali mokrice *P. scaber* preizkusili pet različnih načinov fiksacije in tri različne raztopine barvila ARS ter za interpretacijo izbrali najustreznejši postopek. Za detekcijo kalcija oziroma mineralnih oblik kalcija v vzorcih kutikule različnih razvojnih stadijev marzupijskih mank, v primerjavi s kutikulo odraslih živali, smo izvedli tudi natančnejše analizne metode. Prisotnost kemijskih elementov v kutikuli smo analizirali z energijsko-disperzijsko rentgensko spektroskopijo (EDXS) na vrstičnem EM s poljsko emisijo (FE-SEM). Vzorce smo fiksirali v metanolu, z namenom ohranitve mineralnih komponent, jih posušili na zraku in naprašili z ogljikom. Analizo mineralnih oblik kalcija v kutikuli smo izvedli z Ramanskim spektrometrom, povezanim s konfokalnim fluorescenčnim mikroskopom. Vzorce smo pripravili na tri načine: (i) fiksacija v metanolu ter sušenje na zraku; (ii) fiksacija v metanolu ter priprava parafinskih rezin; (iii) intaktni vzorci brez predhodnega izpostavljanja kemikalijam.

Ugotovili smo, da sta horion in vitelinska membrana pri embrijih mokric *P. scaber* tanjši od jajčnih ovojnic zrelih jajčec iz ovarija vinske mušice *D. melanogaster*. Horion pri mokrici je strukturno enostavnejši, saj je zgrajen iz enega samega homogenega sloja, v primerjavi s horionom vinske mušice, ki je diferenciran v tri sloje: eksohorion, endohorion in notranji horionski sloj. Strukturi vitelinskih membran sta pri obeh vrstah podobni, zgrajeni iz prevladujočega homogenega sloja in površinskega sloja, ki je tanek in naguban. Navedene razlike med ovojnicami teh dveh vrst členonožcev lahko razložimo z dejstvom, da so embriji mokric med razvojem dodatno zaščiteni v valilniku samice, embriji vinske mušice pa so izpostavljeni zunanjemu okolju.

Pokazali smo, da embrionalni epidermis mokrice *P. scaber* med razvojem izloči več apikalnih zunajceličnih matriksov, vsaj dva prekutikularna matriksa in v zadnjem obdobju embriogeneze prvo kutikulo. Ti se zamenjajo v času večjih morfoloških sprememb, ki spremljajo rast in razvoj embrijev ter ličink. Prvo izločanje epidermalnega matriksa smo opazili pri srednjem embriju v stadiju 10, kjer je nad izboklinami apikalne plazmaleme prisoten tanek sloj materiala. Prekutikularna matriksa, ki nastaneta pred formacijo kutikule, se tvorita zaporedno v srednjem embriju in sta po strukturi in sestavi organskega ogrodja drugačna od značilne eksoskeletne kutikule rakov. Sestavljena sta iz rahlega, elektronsko svetlega materiala, ki ga pokriva elektronsko gosta lamina. Iz površine embrija se odluščita med prehodom iz srednjega v pozni embrij (izleganje iz horiona) in med prehodom iz embrionalnega obdobja v obdobje ličinke (izleganje iz vitelinske membrane). Ti matriksi imajo verjetno zaščitno funkcijo ter vlogo regulacije prehoda snovi skozi embrionalno površino in vzpostavitve primernega mikrookolja za formacijo kutikule.

Nastanek prvega kutikularnega matriksa nad epidermisom smo opazili v zadnjem obdobju embriogeneze. Pri poznem embriju v stadiju 18 je kutikula tanka, sestavljena iz epikutikule in prokutikule, ima kutikularne luske in glede na vezavo lektinov WGA vsebuje tudi molekule z *N*- acetilglukozaminom. Do konca embrionalnega razvoja se kutikula odebeli in je strukturno podobna kutikuli odraslih, diferencirana je v epi-, eksо- in endokutikulo s podsloji. Prva levitev poteče približno ob začetku razvoja ličinke. Marzupijska manka izloča novo kutikulo, nadaljnji razvoj pa vodi v povečanje debeline kutikule in strukturno diferenciacijo kutikularnih slojev. Vezava lektinov WGA na kutikulo v tem obdobju larvalnega razvoja je intenzivna, kar kaže, da je organsko ogrodje te kutikule podobno kot v kutikuli odraslih. V skladu z napredovanjem razvoja marzupijskih mank je kutikula po ultrastrukturi in sestavi vedno bolj podobna kutikuli odraslih živali in omogoča tudi gibanje mank, ki smo ga opazili v valilniku. Pred sprostitvijo mank iz valilnika se eksoskeletna kutikula odmakne od epidermisa (apoliza), notranji kutikularni sloji pa se začnejo razgrajevati. Nastane levitveni prostor, v katerega se nad apikalno plazmalemo epidermalnih celic izloča novi kutikula. Iz rezultatov sklepamo, da je marzupijska manka v fazi predlevitve, levi pa se predvidoma kmalu po sprostitvi iz valilnika.

Rezultati histokemijske lokalizacije kalcificiranega tkiva z barvilom alizarin rdeče S so pokazali, da je eksoskeletna kutikula znatno kalcificirana že v stadijih marzupijske manke. Elementne analize z EDXS – FE-SEM kažejo, da se v eksoskeletni kutikuli novo izležene marzupijske manke že akumulira kalcij. Razmerje vrhov kalcija in fosforja je manjše od 1, v primerjavi z značilnim visokim razmerjem pri kutikuli odraslih. Ramanski spektri prisotnosti kalcijevih mineralov v kutikuli novo izležene manke ne pokažejo, iz česar sklepamo, da so pod mejo detekcije Ramanske spektroskopije. Spektri, pridobljeni z EDXS in Ramansko spektroskopijo kutikule naprednih zgodnjih marzupijskih mank in srednjih marzupijskih mank so podobni spektrom kutikule odraslih živali, iz česar sklepamo, da sta elementna in mineralna sestava kutikule v teh stadijih ličink že podobni sestavi kutikule odraslih živali.

Ultrastrukturna organizacija epidermalnih celic je povezana z diferenciacijo epidermisa med razvojem embrijev in mank in s sintezo apikalnih zunajceličnih matriksov. V skladu z zaporednimi razvojnimi stadiji embrijev in mank poteka oblikovanje subapikalnih medceličnih stikov, kar je pokazatelj diferenciacije epitelnih celic, polariziranih v apikalno-bazalni smeri. V stadiju srednjega embrija, pri katerem poteka nastajanje prvega epidermalnega apikalnega matriksa, smo opazili številne delitve epidermalnih celic. Oblikovani so subapikalni adherentni stiki, ki so pomembni pri mehanski povezavi celic in pri vzpostavitvi polarnosti epitela. Septirani stiki, ki so pomembni kot paracelularna pregrada, se oblikujejo pri poznem embriju pred izleganjem, v obdobju tvorbe prve kutikule. Oblika epidermalnih celic se med srednjo in pozno embriogenezo spreminja od cilindrične preko kubične do ploščate. V povezavi

s sintezo in izločanjem apikalnih zunajceličnih matriksov se apikalna plazmalema epidermalnih celic pri različnih razvojnih stadijih preoblikuje v nizke izbokline z elektronsko gostimi vrhovi, pri sintezi nove kutikule pa so prisotni tudi široki citoplazemski izrastki, ki segajo v kutikulo. Epidermalne celice srednjega embrija v stadiju 10 vsebujejo posamezne preproste skladovnice Golgijevega kompleksa, v nadaljnjih stadijih pa so obsežnost, organizacija in razporeditev Golgijevega aparata različne in se spreminjajo verjetno tako glede na diferenciacijo celice kot tudi glede na fazo sinteze apikalnega matriksa.

Strukturno ogrodje mišičnoskeletnih povezav smo analizirali samo pri poznem embriju pred izleganjem iz vitelinske membrane (stadij 19) in pri marzupijskih mankah. Ugotovili smo, da so pri teh razvojnih stadijih že vzpostavljene mišičnoskeletne povezave, kar omogoča gibanje živali znotraj valilnika. Med kutikulo in apikalno plazmalemo tenocita so številna navpično potekajoča povezovalna vlakna. Tenociti so ultrastrukturno podobni celicam pri odraslih in vsebujejo snope mikrotubulov, ki potekajo v apikalno-bazalni smeri. Ultrastruktura stika med tenocitom in mišično celico je tudi v osnovi že podobna strukturi stika pri odraslih živalih. Pokazali smo, da tudi pri menjavi eksoskeleta tenociti ostanejo povezani s staro in novo kutikulo preko navpično razporejenih vlaken, kar velja za analizirane razvojne stadije in za odrasle živali v fazi predlevitve. S tem je omogočeno vsaj osnovno gibanje živali v tem obdobju.

Glede na ultrastrukturno analizo se v zgodnjih fazah tvorba črevesnega apikalnega matriksa med razvojem bistveno ne razlikuje od zgodnje diferenciacije epidermalnih matriksov. Specializirane funkcije obeh matriksov verjetno še niso vzpostavljene tako kot pri odraslih. Pri poznih embrijih v stadijih 16 in 18 je v črevesu prisoten apikalni zunajcelični matriks, ki je strukturno podoben prekutikularnemu matriksu epidermalnih celic. Strukturne razlike med obema kutikulama so v poznejših stadijih razvoja v valilniku večje, saj verjetno obe kutikuli postopoma prevzemata svoji specifični vlogi, eksoskeletna kutikula zaščitno in oporno vlogo, črevesna kutikula pa vlogo pri predelavi, transportu in absorpciji hrane. Pri poznem embriju v stadiju 19 struktura apikalnega matriksa črevesnih celic že kaže podobnost s strukturo črevesne kutikule odraslih živali, vendar diferenciacija slojev še ni razvidna. Pri marzupijskih mankah je vidna nadaljnja diferenciacija črevesne kutikule, vendar ta še ni v tolikšni meri podobna črevesni kutikuli odraslih živali, kot je eksoskeletna kutikula marzupijskih mank podobna eksoskeletu pri odraslih. To morda nakazuje manjši časovni zamik v diferenciaciji črevesne kutikule glede na eksoskeletno kutikulo. Za razliko od odraslih živali je črevesna kutikula marzupijskih mank tanjša, elektronsko gosta plast epikutikule je bistveno manj izrazita, prokutikula anteriorne komore pa še ni diferencirana v podsloje. Kutikularni trni so že prisotni. Pri pozni marzupijski manki so, tako kot pri eksoskeletu, tudi v črevesu opazni znaki levitve kutikule.

4.2 SUMMARY

Exoskeletal cuticle of crustaceans is an apical extracellular matrix of epidermis, based on chitinous organic scaffold and stiffened by sclerotization and calcification. The organic phase is composed of chitin-protein fibers, different proteins and lipids. The mineral phase consists of crystalline calcium carbonate (calcite and Mg-calcite), amorphous calcium carbonate (ACC) and amorphous calcium phosphate (ACP). The cuticle is organized in three principal horizontal layers: epicuticle, exocuticle and endocuticle, which differ in structure, function and molecular composition. New cuticle is formed during development of embryos and larvae and during molting of adult animals. Formation of the new cuticle involves synthesis and elaboration of organic scaffold structure, calcification and establishment of mechanical connections to muscles.

Embryonic development of terrestrial isopods (Oniscidea) takes place in the aqueous environment of marsupium, the brood pouch on the ventral side of female body. Embryos hatch from egg envelopes as larvae mancae, that continue their development in the marsupium for about a week. In different developmental stages embryos are covered by different protective matrices. Egg envelopes cover embryos from the beginning of development, while apical extracellular matrices of epidermis are formed later during development. In arthropods two egg envelopes are present, the outer is chorion and the inner is vitelline membrane. In isopods, osmotic and ionic conditions in embryonic microenvironment are regulated by egg envelopes and dorsal organ of embryo, and in addition, the regulation is partly provided by the composition of marsupial fluid. In embryos of *Porcellio scaber* hatching from the chorion occurs during transition from mid-stage to late-stage embryo, in the period of pronounced morphological changes. Hatching from the vitelline membrane defines the transition from embryo to manca.

The majority of data on the early chitionous matrices formation during embryonic development derives from insects, mainly from the studies of the model species *Drosophila melanogaster*. Data on differentiation of the chitinous matrices during crustacean development are limited and especially ultrastructural studies are rare. Several histological and *in vivo* observations of intact embryos and larvae have been performed in decapods, branchiopods and amphipods. Ultrastructural study of cuticle differentiation was recently performed in embryos of the aquatic amhipod *Parhyale hawaiensis.* From the studies of molting adult crustaceans, embryos and molting larvae of insects, it is known that the shape and ultrastructure of epithelial cells change during cuticle formation. Cuticular components or their precursors are synthesized in the cytosol and in the organelles of the polarized epithelial cells and are then transported across the apical plasma membrane to the extracellular space. Formation of the apical

epidermal matrices during intramarsupial development of isopods have not been investigated in detail yet. Calcification of the chitinous matrices during development is not known. The arthropod exoskeleton is mechanically connected to the muscles by specific attachment of the cuticular matrix to the apical plasma membrane of specialized epithelial cell (tendon cell) and by myotendinous attachment. These attachments involve a complex network of cytoskeletal elements, cell junctions and cell-matrix junctions. Data on formation of structural connection between exoskeleton and muscles during embryonic development of crustaceans are scarce. The apical surface of the gut epithelial cells is also covered by a cuticular matrix. The gut cuticular lining is structurally different from the exoskeletal cuticle and it consists of the outer electron dense epicuticle and the inner more electron lucent procuticle. Differentiation of the gut cuticular matrix during development of crustaceans has not been examined in detail yet.

The main aim of the doctoral dissertation is unraveling of exoskeletal cuticle differentiation during isopod *P. scaber* embryonic development and development of larvae mancae in the marsupium, with emphasis on functional ultrastructure and composition analysis. Ultrastructural characterization of epidermal apical matrices, egg envelopes and gut apical matrices was performed. In addition, the composition of the epidermal apical matrices was analysed, with emphasis on localization of macromolecules containing *N*-acetyl-glucosamine, including chitin, and analysis of the elemental and mineral composition of the cuticle. Next, selected ultrastructural characteristics of epidermal cells, related to development and synthesis of apical matrix, were examined. The ultrastructure of mechanical connections between exoskeleton, specialized epidermal cells (tendon cells) and muscle cells in embryos and marsupial mancae is characterized.

The study was performed in the model organism, terrestrial isopod *P. scaber*. Developmental stages were identified according to Milatovič et al. (2010) developmental staging system of *P. scaber*. As development of larvae in marsupium was not precisely classified before, we described four sequential developmental stages of marsupial mancae. Specimens destined for ultrastructural analyses by transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were fixed in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH = 7.2) and postfixed in 1 % osmium tetroxide. Samples for TEM imaging were embedded in epoxy resin and ultrathin sections were made. Specimens for SEM imaging were chemically dried and coated with gold. Localization of *N*-acetyl-glucosamine containing macromolecules, including chitin, was performed by labelling with lectin WGA ('wheat germ agglutinin'), conjugated to 10-nm gold. Specimens destined for labelling were fixed in 2 % paraformaldehyde and 0.25 % glutaraldehyde in 0.1 M Hepes buffer (pH = 7.2) and then embedded in acrylic resin. Ultrathin sections of embryos and mancae were labelled simultaneously with sections of decalcified cuticle of adults, used as positive control.

Different methods were performed to examine cuticle calcification. First, we localized calcified tissue histochemically by alizarin red S (ARS) staining. Five different methods of fixation and three different staining staining were evaluated on marsupial mancae and adults *P. scaber*. The procedures that resulted in distinct differential staining were used to identified the intensely calcified cuticle. Next, more demanding analytical methods were performed to analyse the presence of calcium and its mineral forms in the cuticle of marsupial mancae in different developmental stages in comparison to cuticle of adult animals. Elemental composition of the cuticle was analysed by energy dispersive X-ray spectroscopy (EDXS) in field-emission-gun scanning electron microscope (FEG-SEM). The samples were fixed in methanol to preserve mineral components, then air-dried and coated with a thin carbon layer. Analysis of the calcium mineral forms in the cuticle was performed by Raman spectrometer, coupled to a confocal fluorescence microscope. The samples were prepared in three different ways: (i) methanol fixation and air-drying; (ii) methanol fixation, paraffin embedding and sectioning; (iii) intact samples without any chemical pretreatment.

We found out that chorion and vitelline membrane of isopod *P. scaber* embryos are thinner in comparison to the egg envelopes of fruitfly *D. melanogaster* mature eggs from ovaries. The horion of *P. scaber* is structurally simpler, composed of a single homogenous layer, in comparison to *Drosophila* horion, which is differentiated in three layers: exochorion, endochorion and inner chorionic layer. Vitelline membranes of both species are similar in structure, consisting of main homogenous layer and a thin and corrugated superficial layer. The established differences in thickness and complexity of egg envelopes between these two species are considered as suitable solutions to development in different environments of embryonic development, *P. scaber* embryos are additionally protected in female marsupium, while *D. melanogaster* embryos are exposed to external environment.

We showed that embryonic epidermis of isopod *P. scaber* secretes several apical extracellular matrices during development, at least two precuticular matrices and, in the last period of embryogenesis, the first cuticle. Renewal of these matrices temporally corresponds to growth and development related morphological changes of embryos and larvae. Early deposition of epidermal matrix was evident in the mid-stage embryo of stage 10, in which a fine, delicate sheet of material is present above the bulges of apical plasma membrane. The precuticular matrices, that precede cuticle formation, are formed successively in the mid-stage embryo. They differ from typical crustacean exoskeletal cuticle regarding their structure and composition of organic scaffold. The precuticular matrix consists of loose, electron lucent material and overlying electron dense lamina. The precuticular matrices are shed during transition from mid-stage to late-stage embryo (hatching from the chorion) and during transition from embryo to manca stage (hatching from the vitelline membrane). Precuticular matrices may participate in

protection of the embryo, regulate the transport of materials through the embryo surface and establish an adequate microenvironment for cuticle formation.

Formation of the first cuticular matrix above the epidermis is evident in the last period of embryogenesis. In late embryo of stage 18 the cuticle is thin and composed of epi- and procuticle. It forms cuticular scales and, regarding lectin WGA binding, contains macromolecules with *N*-acetyl-glucosamine. Until the end of embryonic development the cuticle thickens and displays pronounced differentiation in epi-, exo- and endocuticle with sublayers, structurally similar to the cuticle of adults. The first ecdysis occurs approximately at the beginning of larval development. Marsupial manca secretes a new cuticle and progression of larval development leads to the cuticle thickening and structural differentiation of cuticular layers. Lectin WGA intensely binds to the cuticle of marsupial manca, indicating that organic scaffold in the cuticle is similar to that in the cuticle of adults. According to the progression of marsupial manca development, the cuticle gains similar ultrastructure and composition to the cuticle of adult animals, and is involved in manca mobility, which was observed within the marsupium. Before the release of manca from the marsupium, the cuticle apolysis and disintegration of the inner cuticular layers are observed. The ecdysial space is formed and a new cuticle is deposited above the apical plasma membrane of the epidermal cells. These results indicate that late marsupial manca is in premolt phase and that molting of manca occur shortly after its release from the marsupium.

The results of histochemical localization of calcified tissue with alizarin red S staining show that the exoskeletal cuticle of marsupial manca is already significantly calcified. Elemental analysis by EDXS – FEG-SEM reveals calcium sequestration in the exoskeletal cuticle of newly hatched marsupial manca. The ratio of calcium to phosphorus peaks is below 1, in comparison to the characteristic high ratio, obtained from the cuticle of adults. Raman spectra do not show any presence of calcium minerals in the cuticle of newly hatched manca, as they are probably under detectable limit of Raman spectroscopy. EDXS spectra and Raman spectra, obtained from the cuticle of advanced early marsupial manca and mid-stage marsupial manca are similar to that in adults, indicating that elemental and mineral composition of the cuticle in these manca stages are already similar to that in the cuticle of adults.

Ultrastructural organization of epidermal cells is related to differentiation of epidermis during development of embryos and mancae and to synthesis of apical extracellular matrices. Different subapical cell junctions were identified in successive developmental stages of embryos and mancae and are interpreted as stages of subapical cell junctions formation and thus as the indicator of apico-basally polarized epithelial cells. In mid-stage embryo of stage 10, in which formation of the early epidermal apical matrix takes place, epidermal cells divisions were evident. Subapical adherent junctions are formed,

that provide mechanical connection between the cells and are involved in organization of cell polarity. Septate junctions are formed in prehatching late embryo, in the period of the first cuticle formation. Epidermal cell shape changes from cylindrical through cubical to squamous during mid- and late embryogenesis. Shallow apical plasma membrane bulges with electron dense tips in different developmental stages and broad cytoplasmic projections, invading the newly forming cuticular matrix, are formed in relation to synthesis and secretion of apical extracellular matrices. Simple single stacks of Golgi apparatus were observed in epidermal cells of stage 10 embryo. In the advanced developmental stages, abundance, organization and arrangement of Golgi apparatus are different and probably related to both, to cell differentiation and to apical matrix synthesis.

The structural framework of musculoskeletal attachment was analysed in prehatching embryo of stage 19 and in marsupial mancae. We showed that musculoskeletal attachment complexes are already established in these developmental stages, enabling animal locomotion within the marsupium. Numerous vertical fibrous connections between the cuticle and the apical membrane of tendon cells were evident. Tendon cells ultrastructurally resemble the tendon cells of adults and are characterized by apico-basal arrays of microtubules. The ultrastructure of myotendinous junction is similar to the general structural outline of muscle attachment in adult animals. We showed that mechanical attachments of tendon cells to the old and new cuticle, provided by the vertical fibers, are maintained also during cuticle replacement in premolt adults and in intramarsupial developmental stages, which enables at least basic movements in this period.

The early stages of gut apical matrices formation during development are similar to the early differentiation of epidermal matrices, as identified by ultrastructural analysis. These two matrices are probably not yet engaged in their specialized functions as in adults. In late embryos of stages 16 and 18, an apical extracellular matrix was observed in the hindgut, structurally resembling the precuticular matrix of epidermal cells. Structural differences between the gut and the exoskeletal cuticle are more apparent in the later intramarsupial stages, as probably both cuticles progressively gain their specific functions, protective and supportive function of exoskeletal cuticle and functions in food digestion, transport and absorption of gut cuticle. The structure of the gut apical matrix in the prehatching embryo of stage 19 is more similar to the gut cuticle of adults, although the differentiation of the cuticular layers is not yet distinctive. Further gut cuticle differentiation is evident in marsupial mancae, but in comparison to exoskeletal cuticle, differentation seem to be delayed. Compared to the gut cuticular lining of adults, the gut cuticle of marsupial mancae is thinner and the electron dense layer of epicuticle is less distinctive. The cuticular spines are already evident. The procuticle in the anterior chamber is not yet differentiated in sublayers. The

morphological features of gut cuticle renewal are evident in late marsupial mancae, similarly as observed in the exoskeletal cuticle.

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ZAHVALA

Doktorsko delo je financirala Javna agencija za raziskovalno dejavnost Republike Slovenije (ARRS) v okviru programa mladih raziskovalcev.

V prvi vrsti se zahvaljujem mentoricama Nadi Žnidaršič in Jasni Štrus za priložnost, uvajanje v raziskovalno delo in v vse, kar spada zraven, za neštete dobronamerne nasvete, čas in potrpljenje. Prav tako hvala za uvajanje v pedagoško delo in za vse druge izkušnje, ki sem jih pridobila v teh letih. Zahvaljujem se članicam komisije za temeljit pregled dela, konstruktivne komentarje in spodbudne besede.

Za pomoč, deljenje izkušenj in družbo pri delu sem hvaležna ostalim sodelavcem na katedri, predvsem Milošu Vittori, Magdi Tušek-Žnidarič, Jožici Murko Bulić, Roku Kostanjšku, Lilijani Bizjak Mali, Sari Novak, Katji Zdešar Kotnik, Moniki Kos, Primožu Zidarju in Andreju Blejcu.

Za sodelovanje pri raziskavah, diskusije in prijaznost se zahvaljujem prof. dr. Waltraud Klepal in Danieli Gruber za delo z vrstično elektronsko mikroskopijo ter Kristini Žagar, prof. dr. Miranu Čehu in Andreji Gajovič za možnost uporabe analiznih mikroskopskih metod. Hvala Urbanu Bogataju, ki je veliko prispeval k raziskavam črevesne kutikule.

Hvala sošolcem in sošolkam pri predmetih podiplomskega študija, skupaj se lahko več naučimo.

Sara Novak, Marko Kolenc, Matej Hočevar, Miloš Vittori in Darja Jenko so bili super družba na kongresih, družba za nepogrešljiva kosila in pijače pa poleg nekaterih že omenjenih še Gregor Bračko, Anja Rajbar, Andrej Meglič, Tanja Višnjar, Tina Mesarič, Tea Romih, Tatjana Robič, Mojca Stojan Dolar, Aleksander Gnjidič. Aleksandru še lepa hvala za odpravljanje vseh čudnih računalniških problemov.

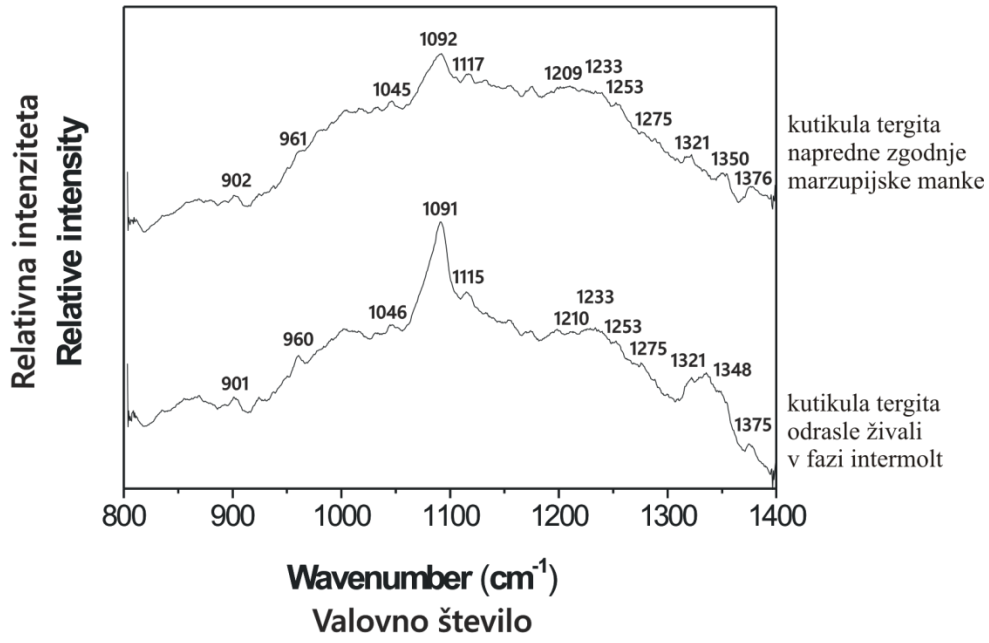
Zahvaljujem se bližnjim domačim za dobronamerne kritike in iskrenost ter za vso potrpežljivost, ki je bila potrebna. Moje Maša, Nika, Maruša in Manca mi dajejo veselje in pogum.

Za iskreno prijateljstvo in sproščen prost čas najlepša hvala Nini Srečnik Lapanja, Nastji Guzzi, Mateji Hafner, Maji Vučnik in Vesni Mladenovi. Hvala Grudnovim šmiklam za vso energijo in pestrost v zadnjih mesecih.

PRILOGE

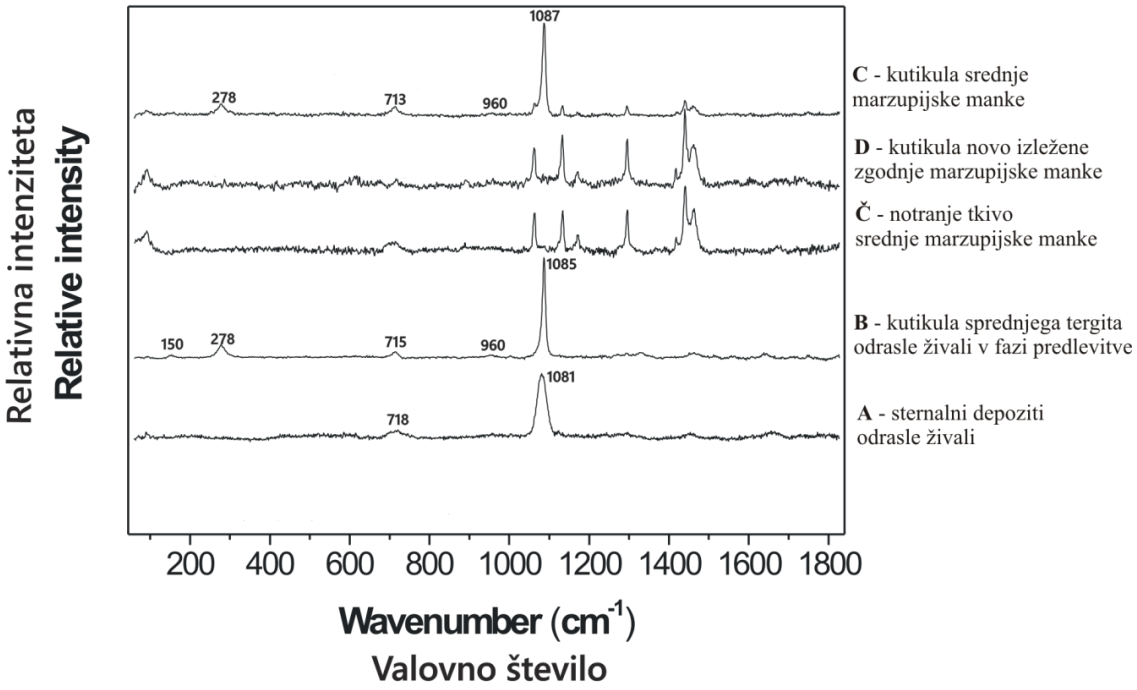
Priloga A

Ramanski spektri kutikule odraslih živali in marzupijskih mank pri raku enakonožcu *Porcellio scaber* (sliki 19 in 20)



Slika 19: Ramanska spektra kutikule odraslih in kutikule napredne zgodnje marzupijske manke raka enakonožca *P. scaber* v območju valovnega števila 800-1400 cm^{-1}. Spodnji spekter: notranja površina kutikule tergita odrasle živali v fazi med levitvama. Zgornji spekter: dorzalna površina tergita napredne zgodnje marzupijske manke. V obeh spektrih sta razvidna dva vrhova, ki sta značilna za karbonat pri 1091 oz. 1092 cm^{-1} in za amorfni kalcijev fosfat (ACP) pri 960 oz. 961 cm^{-1}. Ostali vrhovi kažejo na prisotnost organskih komponent kutikule. Vrhovi, označeni z valovnimi števili, so značilni za hitin.

Figure 19: Raman spectra obtained from the cuticle of adults and the cuticle of advanced early marsupial manca of isopod *P. scaber* in the range of 800-1400 cm^{-1}. The lower spectrum: the inner surface of tergite cuticle of adult intermolt animal. The upper spectrum: the dorsal surface of tergite cuticle of advanced early marsupial manca. Both spectra show two peaks, characteristic for carbonate at 1091 / 1092 cm^{-1} and for amorphous calcium phosphate (ACP) at 960 / 961 cm^{-1}. Other peaks indicate the organic components of the cuticle. The peaks, marked by wavenumber are characteristic for chitin.



Slika 20: Ramanski spektri različnih vzorcev kutikule raka enakonožca *P. scaber* v območju valovnega števila 60-1840 cm^{-1}. Spektri sternalnih depozitov (**A**) in kutikule sprednjega tergita (**B**) odrasle živali v fazi predlevitve kažejo prisotnost kalcijevega karbonata z vrhovi pri okoli 1085 cm^{-1} in okoli 715 cm^{-1}. Vrhova sta pri sternalnih depozitih širša, kar je značilno za amorfni kalcijev karbonat (ACC). Prisotnost amorfnega kalcijevega fosfata (ACP) v kutikuli sprednjega tergita je nakazana z nizkim vrhom pri 960 cm^{-1}. V spektru kutikule srednje marzupijske manke (**C**) prisotnost in oblika vrhov pri 278 cm^{-1}, 713 cm^{-1} in 1087 cm^{-1} kažeta na prevladovanje kalcita, nizek vrh pri 960 cm^{-1} pa na prisotnost majhne količine ACP. Teh vrhov ni v spektru notranjega tkiva srednje marzupijske manke (**Č**) in ne v spektru kutikule novo izležene zgodnje marzupijske manke (**D**).

Figure 20: Raman spectra of different *P. scaber* cuticle samples in the range of 60-1840 cm^{-1} wavenumber. Spectra of sternal deposits (**A**) and anterior tergite cuticle (**B**) in premolt adult animal show presence of calcium carbonate with the peaks at around 1085 cm^{-1} and around 715 cm^{-1}. In the spectrum of sternal deposits the calcium carbonate peaks are wider, which is characteristic for amorphous mineral (ACC). Low peak at 960 cm^{-1} in anterior tergite cuticle shows presence of amorphous calcium phosphate (ACP). In the spectrum of mid-stage marsupial manca cuticle (**C**) the presence and shape of the peaks at 278 cm^{-1}, 713 cm^{-1} and 1087 cm^{-1} show that calcite is the most conspicuous mineral in the cuticle. Low peak at 960 cm^{-1} shows slight presence of ACP. The peaks of mineral components are absent in the spectrum of manca interior tissue (**Č**) and in the spectrum of newly hatched early manca cuticle (**D**).

Priloga B

Dovoljenje založnika Pensoft za objavo člankov "Egg envelopes and cuticle renewal in *Porcellio* embryos and marsupial mancas" in "Exoskeleton anchoring to tendon cells and muscles in molting isopod crustaceans" v tiskani in elektronski verziji svoje doktorske disertacije

Mrak, Polona

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>
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> - Nada Žnidaršič, Polona Mrak, Magda Tušek-Žnidarič, Jasna Štrus: Exoskeleton anchoring to tendon cells and muscles in molting isopod crustaceans. Pages: 39 - 53.
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