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**ZGRADBA SEKRECIJSKIH EPITELIJEV IN MINERALIZACIJA
ZUNAJCELIČNEGA MATRIKSA MED LEVITVIJO
PODZEMELJSKIH IN POVRŠINSKIH MOKRIC (Crustacea: Isopoda)**

DOKTORSKA DISERTACIJA

**STRUCTURE OF SECRETORY EPITHELIA AND
MINERALIZATION OF EXTRACELLULAR MATRIX DURING
MOLT IN SUBTERRANEAN AND EPIGEAN WOODLICE (Crustacea:
Isopoda)**

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Doktorska disertacija je rezultat raziskav, ki so potekale na Oddelku za biologijo Biotehniške fakultete Univerze v Ljubljani. Energijsko-disperzijska rentgenska spektrometrija ter elektronska difrakcija sta bili opravljene na Oddelku za nanostrukturirane materiale Inštituta Jožef Stefan v Ljubljani. Ramanska spektroskopija je bila opravljena v Laboratoriju za strukturo biomolekul Kemijskega inštituta v Ljubljani.

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Doktorsko delo je rezultat lastnega raziskovalnega dela. Podpisani se strinjam z objavo svoje naloge v polnem tekstu na spletni strani Digitalne knjižnice Biotehniške fakultete. Izjavljam, da je naloga, ki sem jo oddal v elektronski obliki, identična tiskani verziji.

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AI Med levitvenim ciklom smo preučili anatomijo, ultrastrukturo in sestavo začasnih depozitov kalcija dveh vrst mokric, podzemeljske *Titanethes albus* ter površinske *Hyloniscus riparius*. Pri *T. albus* smo opisali potek in trajanje levitvenega cikla, ultrastrukturo integumenta med levitvenim ciklom in zgradbo ščetinastih žleznih organov samcev. Levitveni cikel *T. albus* je daljši kot pri površinskih mokricah, ultrastrukturne značilnosti epitelijskega integumenta med sintezo kutikule pa so podobne kot pri drugih rakih. Ščetinasti žlezni organi samcev *T. albus* vsebujejo rozetaste eksokrine žleze, kakršne v literaturi še niso bile opisane. V zadku se nahajata še dva tipa rozetastih eksokrinih žlez, značilnih le za samce. Na ščetinastih žleznih organih so prisotne epikutikularne ščetine in specializirane senzile. Pri obeh obravnavanih vrstah sta prisotna dva para kalcijevih telesc, organov, v katerih se kopiči kalcij. Pri *T. albus* oba para kalcijevih telesc vsebujeta bakterije, pri *H. riparius* pa jih vsebuje le posteriorni par. Pri *T. albus* se pred levitvijo razvijejo sternalni depoziti kalcijevega karbonata, ki jih *H. riparius* nismo zasledili. Matriks kalcijevih telesc se med levitvenim ciklom spreminja, kar nakazuje, da je njihova funkcija povezana s kopičenjem kalcija pred levitvijo in z mineralizacijo kutikule. Matriks kalcijevih telesc, ki vsebujejo bakterije, je mineraliziran s kalcijevim fosfatom in kalcijevim karbonatom, v kalcijevih telescih brez bakterij pa je prisoten le kalcijev karbonat. Ultrastruktura epitelijev kalcijevih telesc kaže na njihovo transportno in sekrecijsko funkcijo. Spreminjanje njihove ultrastrukture med levitvenim ciklom sovпада s transportom kalcija skozi epitelije. Pri *H. riparius* so v medceličnih prostorih epitelijev kalcijevih telesc med resorpcijo kalcija prisotne elektronsko goste granule, ki jih pri *T. albus* nismo opazili.

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AB We studied the anatomy, ultrastructure and composition of transient calcium deposits during the molt cycle in two woodlice, the subterranean *Titanethes albus* and the epigean *Hyloniscus riparius*. In *T. albus* we also monitored the process and duration of the molt cycle, the ultrastructure of the integument during the molt cycle and the structure of gland-piliferous organs of males. The molt cycle of *T. albus* is longer than in epigean woodlice, but the ultrastructure of its integumental epithelium during cuticle synthesis is similar to that reported in other crustaceans. The gland-piliferous organs of *T. albus* males contain exocrine rosette glands unlike any previously reported in available literature. Two more types of male-specific rosette glands are present in the pleon. Epicuticular hairs and specialized sensilla are present on gland-piliferous organs. Two pairs of calcium accumulating organs, the calcium bodies, are present in both species. In *T. albus*, both pairs of calcium bodies contain bacteria, whereas in *H. riparius*, bacteria are present only in the posterior pair. Before molt, *T. albus* forms sternal calcium carbonate deposits, which were not observed in *H. riparius*. The calcium body matrix changes during the molt cycle, indicating that calcium bodies function in calcium accumulation before molt and in the mineralization of the cuticle. The matrix of bacteria-containing calcium bodies is mineralized with calcium phosphate and calcium carbonate, whereas only calcium carbonate is present in bacteria-free calcium bodies. The ultrastructure of calcium body epithelia indicates that they are involved in transport and secretion. Ultrastructural changes of calcium body epithelia during the molt cycle coincide with transepithelial calcium transport. In calcium body epithelia of *H. riparius*, electron dense granules are present in intercellular spaces during calcium resorption, but they were not observed in *T. albus*.

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KRATICE IN OKRAJŠAVE

ACC – amorfni kalcijev karbonat

ACP – amorfni kalcijev fosfat

EDXS – energijsko-disperzijska rentgenska spektrometrija

EELS – spektrometrija izgube energije elektronov

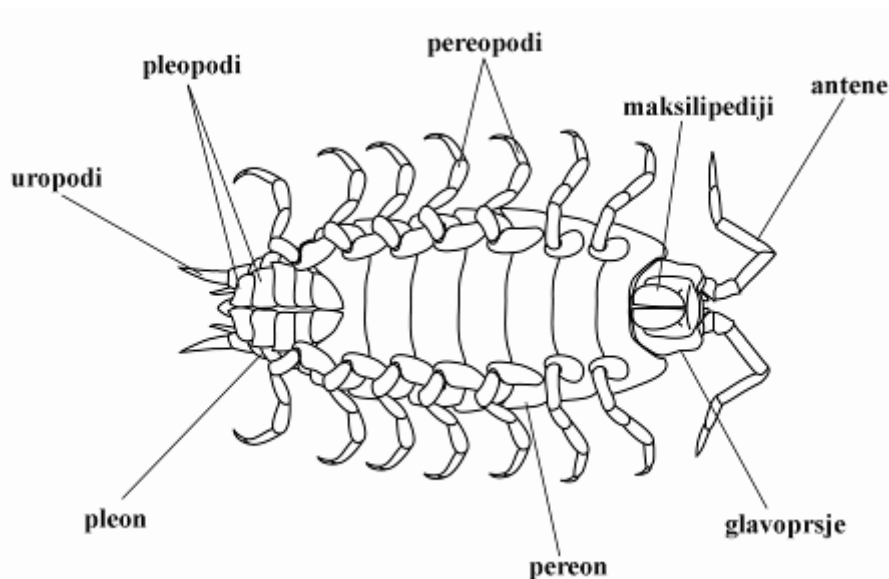
1.1 BIOMINERALIZACIJA IN MINERALIZIRANI MATRIKSI RAKOV

Številni živi organizmi, vključno s človekom, v procesu biomineralizacije nadzorovano izgrajujejo organsko-mineralne strukture z različnimi funkcijami. Mineraliziran matriks pogosto gradi skelet organizmov, jim nudi zaščito in lahko predstavlja zalogo mineralnih snovi, ki sodelujejo v presnovi.

V procesih biomineralizacije pri živalih nastajajo različni minerali, najbolj razširjene pa so kalcijeve soli. Pogosta sta predvsem kalcijev karbonat, ki je razširjen med nevretenčarji, ter kalcijev fosfat, ki je značilen za vretenčarje in ramenonožce (Kawasaki in Weiss, 2006). Drugi minerali zunajceličnih matriksov živali vključujejo silikate (zlasti pri spužvah; Skinner in Jahren, 2003), magnetit (strgača hitonov; Skinner in Jahren, 2003) in nekatere druge minerale (Ehrlich, 2010). Mineralizirani zunajcelični matriksi so sestavljeni iz organskih in mineralnih komponent. Makromolekularne organske komponente matriksov pomembno vplivajo na proces mineralizacije. Sodelujejo pri nukleaciji mineralov in lahko določajo arhitekturo matriksa, kemijsko obliko precipitiranega minerala in usmerjenost kristalov (Ehrlich, 2010).

Telo rakov (Crustacea) pokriva trdna kutikula, ki je pri večini vrst mineralizirana. Ker raki kutikulo redno obnavljajo z levitvijo, so dobri modelni organizmi za študij biomineralizacije (Luquet, 2012). Že pred levitvijo epitelij integumenta izloči nekaj plasti nove kutikule, v katero se po levitvi v kratkem času naložijo minerali, s čimer pridobi potrebne mehanske lastnosti (Neufeld in Cameron, 1993).

Telo rakov je členjeno in njihovi telesni členi se združujejo v telesne regije, kar je splošna značilnost členonožcev. Pri rakah telo običajno sestavljajo glava, oprsje in zadek, vsi ali le nekateri členi oprsja pa se pri nekaterih skupinah združijo z glavo v glavoprsje. Rake enakonožce ali mokrice (Isopoda) prištevamo med višje rake (Malacostraca). Telo mokric je razdeljeno na glavoprsje, ki ga sestavljata glava in pridružen prvi člen oprsja, ter pereon iz preostalih sedmih členov oprsja (slika 1). Zadek sestavlja pet pleonitov in pleotelzon iz zadnjične krpe (telzona), zlite z zadnjim členom pleona. Pri mokricah so hodične okončine pereona (pereopodi) enovejnate in so si med seboj podobne, okončine pleona (pleopodi) pa so dvovejnate in večinoma listaste. Edini par okončin pleotelzona (uropodi) je dvovejnat in pri različnih vrstah različno oblikovan (Wägele, 1992).



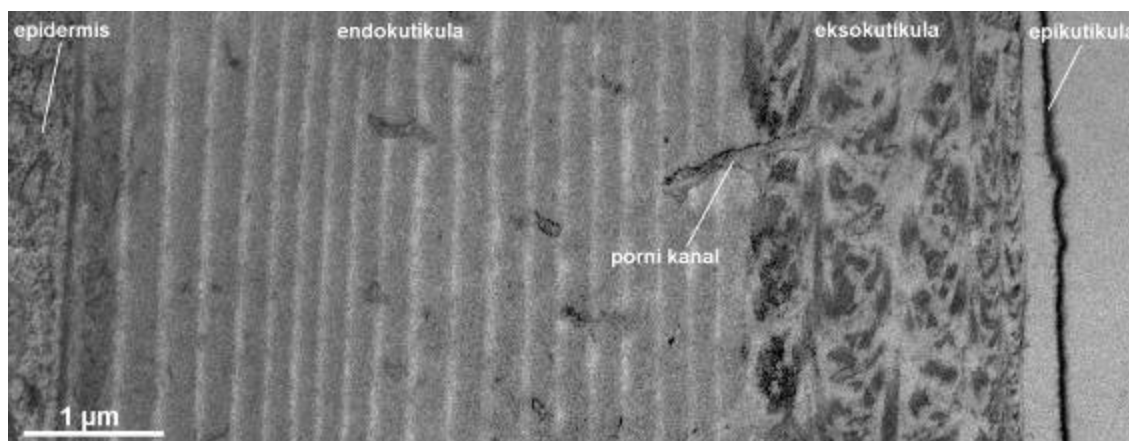
Slika 1: Poenostavljen prikaz telesne zgradbe mokrice s trebušne strani.

Figure 1: A simplified depiction of the body structure of a woodlouse (ventral view).

Kutikula integumenta rakov je običajno troslojna. Strukturo kutikule hrbtne ploščice (tergita) mokrice prikazuje slika 2. Najbolj zunanja plast je tanka epikutikula, ki jo po navedbah nekaterih avtorjev sestavljajo pretežno lipoproteini (Roer in Dillaman, 1984). Pod njo leži debelejša eksokutikula, ki je sestavljena iz hitinsko-proteinskih vlaken in je mineralizirana (Roer in Dillaman, 1984). Raziskave mehanske trdnosti so pokazale, da je eksokutikula rakov deseteronožcev trša od endokutikule (Raabe in sod., 2005). Pod eksokutikulo se nahaja endokutikula, ki je prav tako mineralizirana in je najdebelejši sloj kutikule (Roer in Dillaman, 1984; Hild in sod., 2008). Sestavlja jo več plasti hitinsko-proteinskih fibril, ki so v posameznih plasteh različno usmerjene. Pod endokutikulo je lahko prisoten še nemineraliziran membranski sloj (Skinner 1962, Hild in sod., 2008), ki pri nekaterih vrstah rakov manjka (Pütz in Buchholz, 1991). Pod kutikulo leži enoslojen epitelij, ki sintetizira kutikulo ob levitvi in med sintezo kutikule spreminja svojo obliko in ultrastrukturo. Epitelijske celice iztezajo v kutikulo dolge izrastke, ki se razvejujejo v sistem pornih kanalov (Roer in Dillaman, 1984). V dokončno izoblikovani kutikuli lahko celični izrastki v pornih kanalih degenerirajo (Compère in Goffinet, 1987).

Kutikula rakov je mineralizirana predvsem s kalcijevim karbonatom (CaCO_3), ki pri mokricah sestavlja približno dve tretjini suhe mase kutikule (Neues in sod., 2007; Hild in sod., 2009). Kalcijev karbonat je mineral, ki se pojavlja v različnih oblikah: v amorfni obliki ter v treh različnih kristalnih oblikah – v obliki kalcita, aragonita ali vaterita (Wehrmeister in sod., 2010). V kutikuli rakov prevladujeta amorfni kalcijev karbonat (ACC) ter kalcit, ki zlasti pri morskih vrstah vsebuje precej magnezija (Neues in sod., 2007). Prisotnost ACC v kutikuli rakov je lahko funkcionalna z vidika resorpcije mineralov iz kutikule pred levitvijo, ker je ACC najbolj topna oblika CaCO_3 (Addadi in sod., 2003). V

manjši meri je v kutikuli prisoten kalcijev fosfat (Neues in sod., 2007; Hild in sod., 2008), čeprav novejša raziskava kažejo, da so vsaj pri nekaterih rakih določeni deli integumenta mineralizirani skoraj izključno s kalcijevim fosfatom, npr. prvi par čeljusti deseteronožca *Cherax quadricarinatus* (Bentov in sod., 2012), ter distalni del drugega para pereopodov bogomoščarja *Odontodactylus scyllarus* (Weaver in sod., 2012).



Slika 2: Ultrastruktura kutikule 4. tergita mokrice *Hyloniscus riparius*.

Figure 2: Ultrastructure of the cuticle of the 4th tergite in the woodlouse *Hyloniscus riparius*.

Ultrastruktura in kemijska sestava kutikule mokric sta bili dobro preučeni pri navadnem prašičku *Porcellio scaber*, navadnem pasavčku *Armadillidium vulgare* (Hild in sod., 2008) ter pri veliki jamski mokrici *Titanethes albus* (Hild in sod., 2009). Ultrastruktura kutikule je bila opisana tudi pri pobležni mokrici *Ligia italica* (Štrus in sod., 2008). Kutikulo mokric sestavljajo epikutikula, eksokutikula, endokutikula ter vsaj pri nekaterih vrstah tudi membranski sloj, podobno kot pri drugih vrstah rakov. Analiza sestave kutikule z energijsko-disperzijsko rentgensko spektrometrijo (EDXS) in ramansko spektroskopijo je pokazala, da je glavni mineral v kutikuli preučeni mokric kalcijev karbonat v obliki kalcita in ACC. Kalcit je prisoten le v eksokutikuli, medtem ko je endokutikula mineralizirana z ACC. Porazdelitev fosforja (Hild in sod., 2008) oz. fosfatnih ionov (Hild in sod., 2009) v kutikuli je naspotje porazdelitve kalcita; fosforja je v eksokutikuli malo, njegova količina je večja v endokutikuli, v membranskem sloju pa je zopet manjša. Vrste mokric, ki se pred plenilci branijo z zvijanjem v kroglico, imajo v kutikuli nekoliko večji delež ACC glede na kalcit ter več mineralnih komponent v primerjavi z organskimi kot vrste, ki se branijo z begom ali oprijemanjem podlage (Neues in sod., 2007).

Nadomeščanje kutikule, ki je potrebno za rast, razvoj in regeneracijo, poteka z levitvijo. Med levitvijo raki zamenjajo celotno telesno kutikulo. Novo kutikulo sintetizirajo epiteljske celice epiderma ter epitelijev sprednjega in zadnjega črevesa, ki sta prav tako pokriti s kutikulo. Pred levitvijo se epiderm najprej loči od stare kutikule. Ta proces se imenuje apoliza in z njim med staro kutikulo in epidermom nastane levitveni prostor, s

tekočino napolnjena vrzel. Epitelijske celice nato najprej oblikujejo epikutikulo in za tem eksokutikulo (Roer in Dillaman, 1984). Ti dve plasti nastaneta pred levitvijo. Po levitvi potekata sinteza endokutikule in membranskega sloja ter mineralizacija novonastale kutikule (Roer in Dillaman, 1984).

S presežno elektronsko mikroskopijo so ugotovili, da so na apikalni površini epitelijskih celic med sintezo kutikule prisotna elektronsko gosta področja (Roer in Dillaman, 1993, Ziegler 1997) na kratkih izrastkih plazmaleme (Koulish in Klepal, 1981; Roer in Dillaman, 1993), ki so mesta sinteze organskega matriksa kutikule. Druge opisane ultrastrukturne značilnosti epitelijev med sintezo kutikule so še proliferacija zrnatega endoplazemskega retikuluma in Golgijevega aparata ter povečanje relativne prostornine mitohondrijev v celici (Koulish in Klepal, 1981; Roer in Dillaman, 1993; Ziegler, 1997), med sintezo eksokutikule pa tudi veliko število elektronsko gostih veziklov v apikalni citoplazmi (Koulish in Klepal, 1981; Greenaway in Farrelly, 1991; Ziegler, 1997). Pri desetonožcih je bilo ugotovljeno, da se pred apolizo celice epiderma povišajo in epitelij preide iz kubičnega v visoko prizmatskega (Roer in Dillaman, 1984). Po levitvi se celice zopet sploščijo.

Levitev se v življenju rakov ponovi večkrat in zaradi cikličnega ponavljanja z njo povezanih procesov lahko govorimo o levitvenem ciklu, ki so ga razdelili v arbitrarne faze. Najbolj osnovna je delitev v naslednje štiri faze: 1) predlevitvena faza (»pre-molt stage«), ki traja od apolize do levitve; (2) levitev (»ecdysis«), med katero se žival izvleče iz stare kutikule; (3) polevitvena faza (»post-molt stage«), ki traja od levitve do konca sinteze kutikule ali njene mineralizacije; (4) faza med levitvama (»inter-molt stage«), ko sinteza kutikule ne poteka, kutikula je čvrsta, v njej pa so prisotne vse kutikularne plasti (Skinner, 1962; slovenski izrazi iz Zdešar, 2005).

Za rake enakonožce je značilna dvofazna levitev, v kateri živali najprej levijo posteriorno in nato anteriorno kutikulo (Verhoeff 1940; Messner 1965). Meja med obema deloma kutikule je med četrtem in petim pereonitom. Zaradi te posebnosti levitvenega cikla mokric lahko ob že predstavljenih fazah levitvenega cikla govorimo tudi o fazi medlevitve (»intra-molt stage«; Price in Holdich, 1980b; Zdešar, 2005), ki je kratko obdobje med levitvijo zadnjega ter sprednjega dela telesa. V povezavi z dvofazno levitvijo je sinteza kutikule v sprednjem delu telesa časovno zamaknjena za sintezo v zadnjem delu telesa (Messner, 1965; Ziegler, 1997). V fazi medlevitve v zadnjem delu telesa že poteka sinteza endokutikule, v sprednjem delu pa se še vedno sintetizira eksokutikula (Price in Holdich, 1980b).

Mineralizacija kutikule ob levitvi zahteva veliko količino kalcija v kratkem času. Različni začasni depoziti kalcija, ki so se razvili pri mnogih skupinah rakov, verjetno omogočajo večjo dostopnost kalcija ob levitvi ter predstavljajo hitro dostopen vir kalcijevih ionov

(Luquet in Marin, 2004). Začasni depoziti kalcija, v katere se kalcij nalaga in se iz njih resorbira po potrebi, so zlasti dobro razviti pri sladkovodnih in kopenskih skupinah rakov (Luquet, 2012).

Znan primer začasnih depozitov kalcija so gastroliti v želodcu deseteronožcev (Travis, 1960; Luquet in Marin, 2004). Gastroliti so polkroglaste tvorbe mineraliziranega matriksa, ki v predlevitveni fazi nastanejo v steni želodca in se ob levitvi sprostijo v črevo, kjer se razgradijo. Tvorbo in mineralizacijo matriksa gastrolitov regulira epitelij gastrolitnega diska (Travis, 1960). Gastroliti so mineralizirani z ACC (Bentov in sod., 2010), organske komponente matriksa pa predstavljajo hitin (Shechter in sod., 2008a), proteoglikani (Fernández in sod., 2012) in posebni proteini (Glazer in sod., 2010; Luquet, 2012), za katere je bilo ugotovljeno, da vežejo kalcijeve ione ter inhibirajo rast kristalov CaCO_3 , zaradi česar predpostavljajo, da pripomorejo k stabilizaciji CaCO_3 v amorfni obliki (Shechter in sod., 2008b; Bentov in sod., 2010). Z jedrsko magnetno resonanco je bilo ugotovljeno tudi, da epitelij gastrolitnega diska v matriks gastrolitov izloča intermediate Krebsovega cikla, in sicer fosfoenolpiruvat, 3-fosfoglicerat (Sato in sod., 2011; Akiva-Tal in sod., 2011) in citrat (Akiva-Tal in sod., 2011), ki lahko stabilizirajo kalcijev karbonat v amorfni obliki. ACC lahko stabilizirajo tudi fosfatni ioni (Akiva-Tal in sod., 2011).

Pri nekaterih postranicah (Amphipoda) iz rodov *Orchestia* in *Niphargus* se kalcij kopiči v posteriornih cekih srednjega črevesa (Graf, 1962) v obliki ACC (Raz in sod., 2002), pri rodu *Niphargus* pa se v srednjem črevesu v fazi predlevitve pojavljajo tudi romboedrični skupki kalcita (Graf, 1962; Luquet in Marin, 2004). Mineralni depoziti se v posteriornih cekih pojavijo v predlevitveni fazi, v polevitveni fazi pa se naglo resorbirajo (Graf, 1962; Graf in Meyran, 1985).

Iz depozitov v posteriornih cekih so izolirali orchestin, specializiran protein, ki veže kalcij, in ga v njih tudi lokalizirali s specifičnimi protitelesi (Testeniére in sod., 2002; Hecker in sod., 2004). Za vezavo kalcija je potrebna fosforilacija proteina, kalcij pa se veže na njegove fosfoserine (Hecker in sod., 2003). Ekspresija orchestina je povečana v fazi predlevitve, ko se v posteriornih cekih oblikujejo depoziti kalcijevega karbonata (Hecker in sod., 2004), v zaznavni količini pa se izraža zgolj v posteriornih cekih in ne v drugih tkivih (Testeniére in sod., 2002). Trenutno pri drugih skupinah rakov ni znan noben protein, katerega aminokislinsko zaporedje bi bilo podobno orchestinovemu in evlucijski izvor orchestina je nepojasnen (Testeniére in sod., 2002).

Naslednji primer razmeroma dobro preučenihi začasnih depozitov kalcija so sternalni depoziti CaCO_3 kopenskih rakov enakonožcev (Oniscidea). Najbolje raziskani so sternalni depoziti navadnega prašička *Porcellio scaber*.

Sternalni depoziti se oblikujejo v predlevitveni fazi v levitvenem prostoru prvih štirih

sternitov pereona (Messner, 1965; Steel, 1993). Sternalne depozite v distalnem delu levitvenega prostora (bližje stari kutikuli) sestavljajo mineralizirane sferule velikosti okoli 1 μm (Ziegler, 1994), v proksimalnem delu levitvenega prostora (bližje epidermu) pa neprekinjena homogena plast mineraliziranega matriksa (Ziegler, 1994). Tako sferule kot homogeni matriks so mineralizirani z ACC (Ziegler, 1994; Becker in sod., 2003). Organske komponente matriksa sternalnih depozitov tvorijo krožne plasti in mrežo radialnih vlaken, debelih manj kot 20 nm (Fabritius in sod., 2005). Novejše raziskave z vrstično elektronsko mikroskopijo kažejo, da so med fazo predlevitve sferule kalcijevega karbonata v levitvenem prostoru prisotne tudi drugje v sprednjem delu telesa, npr. na hrbtni strani (Neues in sod., 2011). Na to možnost je nakazal že Steel (1993).

Začasne depozite kalcija pri rakih praviloma sestavlja ACC. Več avtorjev (Raz in sod., 2002; Becker in sod., 2003; Addadi in sod., 2003) je predpostavilo, da je amorfna oblika CaCO_3 v začasnih mineraliziranih strukturah funkcionalna zaradi večje topnosti amorfnega CaCO_3 v primerjavi z njegovimi kristalnimi oblikami, t.j. kalcitom, vateritom in aragonitom.

1.2 TRANSPORT KALCIJA SKOZI EPITELIJ

Tvorba in razgradnja mineraliziranih matriksov rakov, tako kutikule integumenta kot matriksov začasnih kalcijevih depozitov, sta funkciji sekrecijskih epitelijev. Poleg sinteze in sekrecije organskih komponent matriksa ti epiteliji tudi regulirajo mineralizacijo ter demineralizacijo omenjenih matriksov. Po levitvi poteka mineralizacija novega eksoskeleta in sočasno (običajno) resorpcija začasnih kalcijevih depozitov, pred naslednjo levitvijo pa resorpcija kalcija iz eksoskeleta ter kopičenje kalcijevih mineralov v začasnih depozitih (Luquet in Marin, 2004; Shechter, 2008a). Tudi epiderm med levitvijo opravlja funkcijo sekrecijskega epitelija, ki izloča in oblikuje organski matriks kutikule ter regulira njeno mineralizacijo. Ti procesi zahtevajo, da epiteliji integumenta in založnih struktur omogočajo intenziven ionski transport v fazah levitvenega cikla, v katerih poteka obsežno premeščanje kalcija.

Transport kalcija skozi epitelij lahko poteka na dva načina: s transcelularnim transportom, pri katerem kalcij vstopa v celice preko transportnih proteinov in se preko njih izloča na nasprotni strani celic, ali s paracelularnim transportom skozi medcelične prostore med epitelijskimi celicami v smeri padajočega koncentracijskega gradienta (Hubbard, 2000; Ziegler in sod., 2005). Prednosti aktivnega transcelularnega transporta so, da je selektiven in lahko teče tudi proti koncentracijskemu gradientu, vendar je zanj potrebna energija in se lahko zasiči. Paracelularni transport je manj selektiven, usmerja ga padajoči koncentracijski gradient in ne zahteva dodatne energije (Hubbard, 2000).

Ker je dvig koncentracije Ca^{2+} tudi znotrajcelični signal in visoka koncentracija Ca^{2+} v

citosolu lahko deluje citotoksično, se morajo epitelijske celice ob transportu velikih količin kalcija izogniti njegovim celično-fiziološkimi učinkom (Simkiss 1996). Problematičnost povišanja citosolne koncentracije Ca^{2+} izhaja iz vloge Ca^{2+} v apoptozi ter iz celične energetike, ki temelji na fosfatnih spojinah. Visoka koncentracija Ca^{2+} lahko vodi v tvorbo kristalov kalcijevih fosfatov zaradi tvorbe soli s fosfatnimi ioni, ki se sproščajo ob hidrolizi visokoenergijskih organskih fosfatov v celičnem metabolizmu (Simkiss, 1996; Clapham, 2007). Poleg tega kalcijevi ioni zelo počasi difundirajo skozi citosol (Hubbard, 2000), transport skozi celico pa bi zahteval visok koncentracijski gradient Ca^{2+} znotraj citoplazme, da bi Ca^{2+} difundiral v določeni smeri skozi celotno celico (Simkiss, 1996).

Eden od možnih načinov transcelularnega transporta je transport znotraj citosola, pri katerem bi bil kalcij vezan na proteine (Simkiss, 1996; Hubbard, 2000). Takšna pot je bila predlagana zlasti pri vretenčarskih modelih, npr. pri ameloblastih med tvorbo zobne sklenine (Hubbard, 2000). Vloga citosolnega prenašalca kalcija je bila pripisana proteinu kalbindinu, ki z visoko afiniteto veže kalcij in se močneje izraža v tkivih, v katerih poteka intenziven transport kalcija. Povečanje izražanja kalbindina časovno sovпада s povečano intenzivnostjo transporta kalcija skozi epitelij (Hubbard, 2000). Druga možnost je organelna pot transporta, pri kateri bi se kalcijevi ioni privzemali v cevaste organele (zlasti gladki endoplazemski retikulum) in difundirali skozi lumen organela na nasprotni pol celice, kjer bi zapustili organel in se izločili iz celice. Tretja predlagana pot, ki je pri večini preučeni modelov slabo podprta, je vezikularni transport kalcija s transcitozo (Simkiss, 1996).

Večina razpoložljivih podatkov o transportu kalcija skozi epitelije pri rakih je bila pridobljena med resorpcijo kalcija iz začasni depozitov ali kutikule. Mnogo manj je podatkov o transportu kalcija skozi epitelije med nasprotnim procesom, mineralizacijo zunajceličnih matriksov.

Razmeroma malo je znanega o mehanizmu transporta kalcija skozi epitelij gastrolitnega diska. Ugotovili so, da se v celicah epitelija gastrolitnega diska kalcij kopiči v mitohondrijih, kar bi bilo lahko povezano z uravnavanjem koncentracije kalcijevih ionov v citosolu med transportom (Ueno in Mizuhira, 1983). Mehanizem transporta kalcija skozi epitelij gastrolitnega diska ostaja nepojasnen (Greenaway in Farrelly, 1991). Ker se gastroliti ob levitvi sprostijo v prebavilo raka, kjer se raztopijo in resorbirajo (Travis, 1960), opravlja epitelij gastrolitnega diska predvsem funkcijo mineralizacije matriksa, ne pa tudi intenzivne resorpcije mineralov, zato je težko primerljiv z epiteliji, ki tvorijo drugečasne depozite kalcija in so dobro preučeni predvsem med njihovo resorpcijo.

Pri amfibijski rakovici *Holthuisana transversa* sta Greenaway in Farrelly (1991) preučila ultrastrukturo epiderma med resorpcijo kalcija iz kutikule in njegovo vezavo v kalcificirane granule, ki se kopičijo v vezivu in hemolimfi. Pri tej vrsti nastajajo v

medceličnih prostorih od 0,8 μm do nekaj μm velike elektronsko goste granule, ki so med nastankom povezane z izvihki lateralne membrane epiteljskih celic in se kopičijo v bližini epiderma. Poleg teh so prisotne tudi podobno velike znotrajcelične granule in manjše (okoli 0,3 μm velike) granule v hemolimfi. Z EDXS sta Greenaway in Farelly pokazala, da vsebujejo vsi trije tipi granul veliko kalcija, v velikih zunajceličnih granulah pa je tudi veliko fosforja. Avtorja zagovarjata transcelularno pot transporta kalcija skozi epiderm ob resorpciji iz kutikule.

Med tvorbo depozitov v posteriornih cekih postranice *Orchestia* oblikujejo celice epitelija posteriornih cekov številne apikalne mikrovile, bazolateralna plazmalema epiteljskih celic pa z nagubanjem tvori omrežje razširjenih medceličnih prostorov med sosednjimi celicami (Graf, 1971). Predlagali so, da se Ca^{2+} koncentrira v medceličnih prostorih preko $\text{Na}^+/\text{Ca}^{2+}$ izmenjevalca (Meyran in sod., 1984). V medceličnih prostorih epitelija posteriornih cekov so v polevitveni fazi prisotne okoli 2 μm velike kalcificirane granule (sferiti), ki nastajajo subapikalno v medceličnih prostorih na izvihkih membrane (pedicelih; Graf in Meyran, 1985). Granule se nato pomaknejo proti bazalnemu delu medceličnega omrežja, kjer razpadejo. Z imunolokalizacijo so pokazali, da je tudi v zunajceličnih granulah prisoten orchestin, protein, ki je sicer sestavni del matriksa mineralnih depozitov v posteriornih cekih (Hecker in sod, 2004).

Graf in Meyran (1985) sta predlagala paracelularno pot kalcija skozi epitelij posteriornih cekov postranic v apikalno-bazalni smeri v polevitveni fazi, ko poteka resorpcija kalcija. Pri tem naj bi kalcijevi ioni prehajali po medceličnem prostoru in se vezali na matriks nastajajočih granul, ki ga na površini membranskih pedicelov izločajo celice. Z vezavo kalcija v granule bi se ohranjala nizka koncentracija kalcijevih ionov v medceličnem omrežju, kar bi omogočalo nadaljnjo resorpcijo kalcija iz depozitov.

Veliko podatkov je na razpolago o ultrastrukturnih značilnostih anteriornega sternalnega epitelija mokric, zlasti navadnega prašička, v povezavi s tvorbo in razgradnjo sternalnih depozitov (Ziegler in sod., 2005). Ta epitelij je za raziskave še posebej primeren zaradi možnosti primerjave s posteriornim sternalnim epitelijem, t.j. epitelijem sternitov v zadnjem delu telesa, kjer se veliki sternalni depoziti ne oblikujejo. Na podlagi primerjave anteriornega in posteriornega sternalnega epitelija lahko sklepamo na to, katere značilnosti epitelijev so verjetno povezane z izgradnjo in resorpcijo sternalnih depozitov.

V predlevitveni fazi, ko epitelij izgrajuje sternalne depozite, se bazolateralna membrana močno naguba in med celicami anteriornega sternalnega epitelija nastane omrežje razširjenih medceličnih prostorov, ki so ga v tem primeru poimenovali intersticijsko omrežje (Ziegler, 1996). V fazi medlevitve (po posteriorni levitvi) se z gubanjem izrazito poveča tudi površina apikalne plazmaleme epiteljskih celic. V intersticijskem omrežju se pojavijo majhne elektronsko goste granule, ki v premeru merijo okoli 100 nm in nastajajo

na izvihkih plazmaleme (Ziegler, 1996). Za te granule je bilo z EDXS (Ziegler, 2002) ter spektroskopijo izgube energije elektronov (EELS; Ziegler, 1996) ugotovljeno, da vsebujejo veliko kalcija, fosforja in dušika. Tako med sintezo kot med razgradnjo sternalnih depozitov se močno poveča prostorninski delež mitohondrijev v celicah anteriornega sternalnega epitelijskega (Glötzner in Ziegler, 2000).

Presenetljivo je bilo poznejše odkritje, da je pri vrstah *Ligia oceanica* in *Ligidium hypnorum* apikalna plazmalema anteriornega sternalnega epitelijskega močno nagubana in so v intersticijskem omrežju prisotne enake elektronsko goste granule tako v predlevitveni fazi kot v fazi medlevitve, torej med sintezo in razgradnjo sternalnih depozitov (Glötzner in Ziegler, 2000). To nakazuje na podobne mehanizme transporta kalcija v smeri od apikalne proti bazalni strani epitelijskega med resorpcijo depozitov in v obratni smeri med njihovo mineralizacijo. Glede tega problema ni bilo nadaljnje delo. Glötzner in Ziegler (2000) sta na podlagi tega rezultata predpostavila, da je transport kalcija v apikalni smeri pri teh dveh vrstah manj učinkovit zaradi njune manjše prilagojenosti na kopensko okolje ter da granule zgolj vzdržujejo dovolj nizko koncentracijo prostih kalcijevih ionov v intersticijskem omrežju in ne pripomorejo k transportu kalcija v levitveni prostor.

Transport preko sternalnega epitelijskega med razgradnjo sternalnih depozitov rakov enakonožcev naj bi bil pretežno transcelularen (Glötzner in Ziegler, 2000; Ziegler in sod., 2005). Študije s precipitacijo kalcijevega oksalata *in vivo* kažejo, da se kalcij med transportom koncentrira v endoplazemskem retikulumu epitelijskih celic (Hagedorn in Ziegler, 2002). Pri transportu verjetno sodeluje Ca^{2+} -ATPaza gladkega endoplazemskega retikuluma (SERCA; Hagedorn in Ziegler, 2002), za katero je bilo s hibridizacijo *in situ* ugotovljeno, da je njeno izražanje povečano v pozni fazi predlevitve ter v fazi medlevitve, torej v fazah, ko poteka intenziven transport kalcija skozi anterioni sternalni epitelij (Hagedorn in sod., 2003). Z do sedaj uporabljenimi metodami niso ugotovili, da bi se kalcij kopičil v mitohondrijih (Ziegler, 2002), kot so pokazali v epiteliju gastrolitnega diska deseteronožcev (Ueno in Mizuhira, 1983). Imunska lokalizacija H^+ -ATPaze tipa V je pokazala, da je ta prenašalec v različnih fazah levitvenega cikla prisoten v mnogo večji gostoti bodisi na apikalni bodisi na bazolateralni plazmalemi celic anteriornega sternalnega epitelijskega (Ziegler in sod., 2004). V predlevitveni fazi se H^+ -ATPaza tipa V nahaja skoraj izključno na bazolateralni plazmalemi epitelijskih celic. V fazi medlevitve, torej med razgradnjo sternalnih depozitov, velja nasprotno; H^+ -ATPaza je lokalizirana skoraj izključno na apikalni plazmalemi celic anteriornega sternalnega epitelijskega. Velika količina H^+ -ATPaze na apikalni plazmalemi kaže na intenziven transport protonov v levitveni prostor. Ta je verjetno povezan za raztapljanjem CaCO_3 v sternalnih depozitih, pri čemer kopičenje protonov zakisa okolje in omogoča sproščanje Ca^{2+} ter HCO_3^- ionov, ki se lahko transportirajo (Ziegler in sod., 2004).

Pri transportu kalcijevih ionov verjetno sodelujeta tako Ca^{2+} -ATPaza kot $\text{Na}^+/\text{Ca}^{2+}$

izmenjevalec, saj je njuno izražanje v anteriornem sternalnem epiteliju je v predlevitveni fazi in v fazi medlevitve povečano v primerjavi s fazo med levitvama (Ziegler in sod., 2002, 2005).

1.3 POVRŠINSKI IN PODZEMELJSKI PREDSTAVNIKI DRUŽINE TRICHONISCIDAE

Kopenski enakonožci (Oniscidea) so najverjetneje monofiletska skupina rakov enakonožcev (Isopoda). Razdelimo jih lahko v 5 večjih filogenetskih linij (Schmidt, 2008). To so Ligiidae, Tylidae, Mesoniscidae (te tri linije so hkrati tudi družine), Synocheta in Crinocheta; trenutno uveljavljen pogled na odnose med njimi kaže slika 3. Vse našteje linije razen skupine Ligiidae skupaj sestavljajo skupino Holoverticata, znotraj katere Mesoniscidae, Synocheta in Crinocheta skupaj tvorijo skupino Orthogonopoda. Nekateri avtorji namesto skupine Ligiidae od ostalih oddeljujejo skupino Tylidae; v tem primeru Ligiidae skupaj s skupino Orthogonopoda sestavljajo skupino Ligiamorpha (Schmidt, 2008). Glede monofilije skupine Orthogonopoda vlada konsenz, ni pa enotnega pogleda na odnose med skupinami znotraj nje. Dva poskusa molekularne filogenije mokric (Michel-Salzat in Bouchon, 2000; Mattern, 2003) sta se osredotočila predvsem na skupino Crinocheta. Obe raziskavi sta vključili predstavnike vseh filogenetskih linij kopenskih enakonožcev razen mesoniscidov (to družino sestavljata le dve vrsti rodu *Mesoniscus*), vendar nista pripomogla k razrešitvi filogenetskih odnosov med večjimi skupinami.

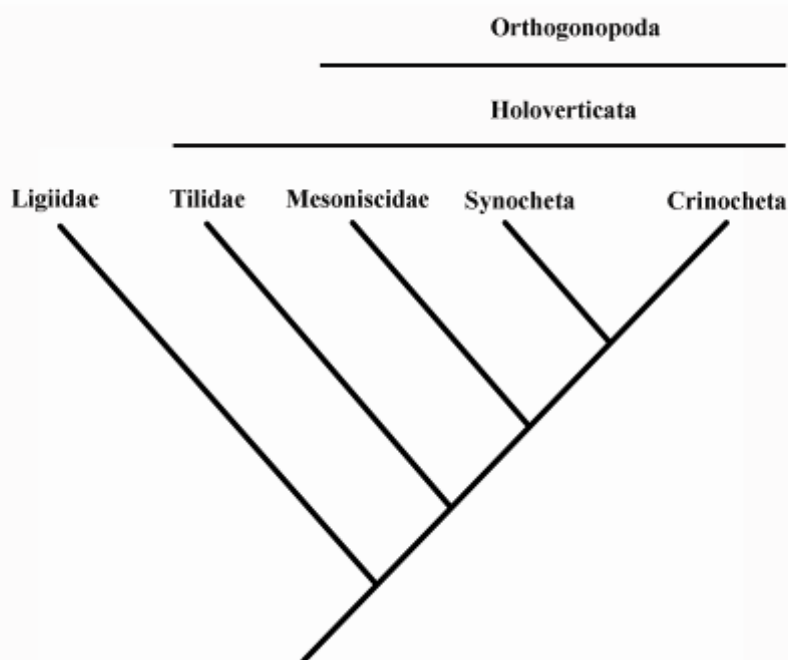
Med naštetimi skupinami je najbolj pestra skupina Crinocheta (Tej skupini pripada okoli 80% opisanih vrst oniscidov; Schmidt, 2008), ki je tudi najbolj preučena. Predstavniki te skupine so zelo dobro prilagojeni na kopensko okolje. Pri njih je npr. razvit zaprt tip valilnika in pri številnih predstavnikih so na pleopodih prisotna trahealna pljuča (Schmidt, 2008).

Družina trihoniscidov (Trichoniscidae) sodi v skupino Synocheta. Trihoniscidi so vezani na vlažna okolja in so večinoma drobne živali, velike le nekaj milimetrov. V skupini so poleg površinskih kopenskih predstavnikov tudi amfibijske, sekundarno vodne in podzemeljske vrste (Schmidt, 2008). Biologija skupine Synocheta je v primerjavi z drugimi skupinami mokric, zlasti skupinama Crinocheta in Ligiidae, slabo raziskana. O strukturnih in kemijskih lastnostih kutikule ter levitvenih cikličnih trihoniscidov podatkov skoraj ni na razpolago; izjema je le velika jamska mokrica *Titanethes albus*. Njena kutikula je bila nedavno raziskana z elektronsko mikroskopijo ter ramansko spektroskopijo (Hild in sod., 2009).

Čeprav skupina Synocheta predstavlja le dobro šestino vseh vrst kopenskih enakonožcev (Schmidt, 2008), predstavljajo trihoniscidi prepričljivo večino (okoli 70 %) vseh do sedaj znanih obligatno jamskih (troglobiontskih) oniscidov (Manicasterri in Argano, 1989). Kot

kaže, so trihoniscidi jamsko okolje kolonizirali uspešneje od drugih skupin mokric.

Manicasteri in Argano (1989) sta kot značilnosti troglobiontov naštel redukcijo oči in kutikularnih pigmentov, razvoj daljših okončin, tanjšanje eksoskeleta, specializacijo čutil, zmanjšanje intenzivnosti metabolizma, zmanjšanje števila potomcev, izgubo letnih in cirkadianih ritmov ter življenje izključno v jamah. O prisotnosti večine teh lastnosti pri jamskih mokricah ni podatkov. Procesi levitve pri jamskih živalih še niso raziskani, primerjalnih študij integumenta pa je le malo (pri mokricah nobene). Študija zgradbe in sestave kutikule velike jamske mokrice (Hild in sod., 2009) ni vključila bližnjih sorodnikov te vrste (drugih trihoniscidov), kar otežuje interpretacijo rezultatov. Nekateri drugi podatki o integumentu jamskih členonožcev, kot je domneva o tanjših kutikulah jamskih živali, so špekulativni ali omejeni na slabo podprte subjektivne ocene (Studier in sod., 1987; Shear in Humphreys, 1996).



Slika 3: Uveljavljena filogenija skupine Oniscidea, kot jo je predstavil Schmidt (2008).

Figure 3: The established phylogeny of Oniscidea, as presented by Schmidt (2008).

Pri večih predstavnikih trihoniscidov so bila v notranjosti pereona opisana kalcijeva telesca (»Kalkreservekörper«; Verhoeff, 1926, 1927, 1931; Méhelý, 1932). Gre za cevaste epitelijske strukture, ki vsebujejo veliko kalcija (Ziegler, 2003). Méhelý (1932) omenja, da so te strukture morda prisotne tudi izven trihoniscidov pri vrstah *Mesoniscus graniger* (Mesoniscidae) ter *Ligidium hypnorum* (Ligiidae), vendar je prisotnost teh organov pri *L. hypnorum* dvomljiva (osebno opažanje na podlagi histološkega pregleda). Kalcijeva telesca še niso bila opisana na ultrastrukturnem nivoju in ni znano, kako se njihova zgradba

spreminja med levitvenim ciklom, zaradi česar težko sklepamo na njihovo funkcijo.

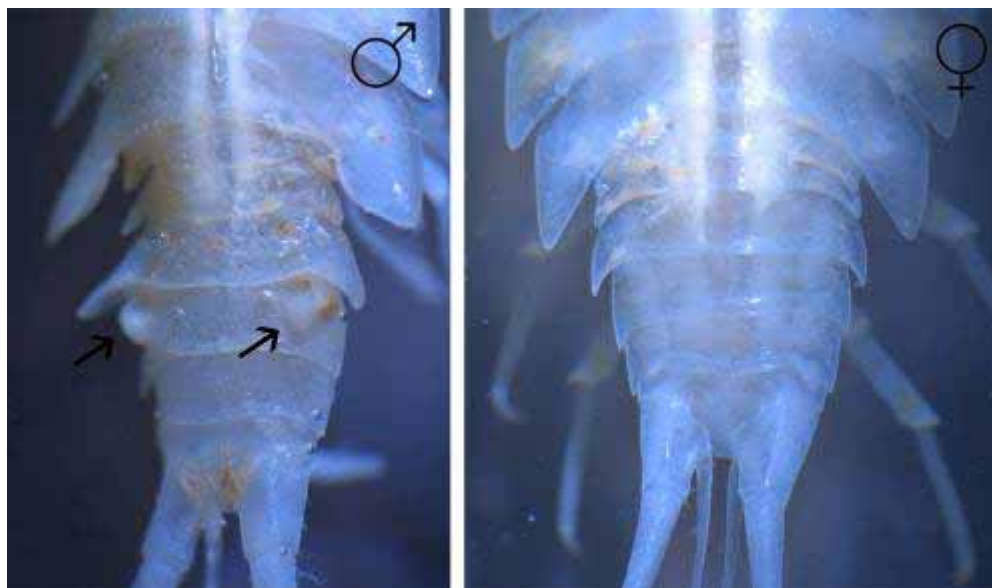
1.4 ŠČETINASTI ŽLEZNI ORGANI

Za številne trihoniscide ter redke predstavnike skupine Crinocheta (rodova *Bethalus* in *Colombophiloscia*) so značilni posebni žlezni organi, ki so prisotni zgolj pri samcih (Tabacaru, 1996). V literaturi v angleškem jeziku so za njih običajno uporabljali izraz »gland-piliferous organs« (Tabacaru, 1996; Taiti in sod., 2011; Montesanto in sod., 2011) kot prevod francoskega izraza »organes glandulo-pilifères«, ki ga je uvedel Vandel (1951). V tem delu bodo poimenovani ščetinasti žlezni organi. Organi so lahko bodisi izbokline bodisi ugreznitve telesne površine. V predelu ščetinastega žleznega organa je površna kutikule gosto oščetinjena, s svetlobno mikroskopijo pa so v kutikuli pogosto opazili številne pore, za katere predpostavljajo, da so izvodila eksokrinih žlez (Vandel, 1951). Ščetinasti žlezni organi so prisotni bodisi na antenah bodisi na dorzalni površini telesa (glavoprsja, pereona ali pleona), vendar vedno le na enem ali dveh členih (Tabacaru, 1996). Funkcija ščetinastih žleznih organov je neznana, najverjetneje pa je povezana z razmnoževanjem (Strouhal, 1939a, 1939b; Lombardo, 2006). Vandel (1951) je predpostavil, da ščetinasti žlezni organi izločajo hlapne snovi in da močna oščetinjenost površine pospešuje izhlapevanje domnevnih feromonov.

Ščetinasti žlezni organi so dobro razviti pri številnih troglobiontskih trihoniscidih, npr. pri rodovih *Titanethes*, *Cyphonethes* (Strouhal, 1939a) in *Trichoniscus* (Vandel, 1951; Tabacaru, 1996; Lombardo in sod., 2006).

Do sedaj so bili ščetinasti žlezni organi opisani na mikroskopskem nivoju le pri jamskem trihoniscidu *Trichoniscus alexandrae* (Lombardo in sod., 2006). Pri tej vrsti je ena oščetinjena površina prisotna mediano na dorzalni strani glavoprsja, druga pa mediano na dorzalni strani prvega pereonita. Med sploščenimi ščetinami so prisotne senzile in pore, ki so izvodila številnih žlez, ki se nahajajo med črevesom in dorzalno površino telesa. Žleze sestavljajo tri zaporedno povezane celice: velika sekrecijska celica, vmesna celica in kanalska celica. Velika sekrecijska celica je napolnjena s sekrecijskimi vezikli z nehomogeno elektronsko gosto vsebino, cevaste ugreznitve njene plazmaleme pa vodijo v kanal, ki poteka skozi vmesno celico. Ta celica vsebuje elektronsko redke vezikle, kanal, ki teče skozi njo, pa se nadaljuje v izvodilo, ki ga oblikuje kanalska celica. Slednja vodi sekrecijske produkte na površino telesa. V veliki sekrecijski celici ter vmesni celici sta dobro razvita zrnati endoplazemski retikulum in Golgijev aparat.

Pri veliki jamski mokrici *Titanethes albus* so ščetinasti žlezni organi prisotni na pleonu (Schiödte, 1849; Strouhal, 1939a). Na zunaj so videti kot parne izbokline (tuberkli) na četrtem členu pleona, ki jih deloma prekrivata povečani epimeri tretjega člena pleona (slika 4). Čeprav se je že zgodaj ugibalo o njihovi žlezni naravi (Verhoeff, 1917; Strouhal, 1939a,b), o žlezah, ki bi izločale na površino izboklin na pleonu samcev, do sedaj ni bilo na voljo nobenih podatkov.



Slika 4: Ščetinasti žlezni organi velike jamske mokrice. Levo pleon samca, desno samice. Puščice kažejo na ščetinaste žlezne organe.

Figure 4: The gland-piliferous organs of *Titanethes albus*. Left: male pleon; right: female pleon. Gland-piliferous organs are indicated by arrows.

1.5 RAZISKOVALNA VPRAŠANJA

Predmet naše raziskave so levitev, sekrecijski epitelijski in mineralizirani zunajcelični matriksi predstavnikov družine Trichoniscidae. Izbrana modelna organizma (slika 5) sta velika jamska mokrica *Titanethes albus* (Koch) in površinski trihoniscid *Hyloniscus riparius* (Koch).

Potek levitve, ultrastrukturni vidiki sinteze kutikule in kopičenje kalcija v levitvenem ciklu pri trihoniscidih še niso bili preučeni. Prav tako do sedaj ni bilo podatkov o sintezi kutikule pri jamskih rakah. Raziskave velike jamske mokrice lahko zato prispevajo pomembne nove podatke o biologiji jamskih organizmov.

Trihoniscidi so za raziskave še posebej privlačni zaradi do sedaj neraziskanih depozitov kalcija, kalcijevih telesc, ki so prisotna pri tej družini. Raziskave teh organov lahko

prinesejo nova odkritja na področjih biomineralizacije in transporta kalcija skozi epitelije. Ultrastrukturnih podatkov o kalcijevih telescih ni na voljo, nekaj opazovanj s svetlobno mikroskopijo na parafinskih rezinah je opravil le Méhely (1932). Funkcije kalcijevih telesc do sedaj niso ugotovili, podatkov o spreminjanju mineraliziranega matriksa v njih pa ni na voljo.

Poleg omenjenih struktur in procesov smo želeli pri jamski mokrici raziskati tudi ščetinaste žlezne organe na zadkih samcev. Ultrastrukturne raziskave teh organov nudijo vpogled v njihovo delovanje in funkcijo v jamskem okolju, omogočajo pa nam tudi primerjavo z drugimi vrstami, na podlagi katere lahko sklepamo ali so ti organi pri različnih rodovih mokric homologni ali pa so se razvili s konvergentno evolucijo.



Slika 5: *Titanethes albus* (zgoraj) in *Hyloniscus riparius* (spodaj).
Figure 5: *Titanethes albus* (top) and *Hyloniscus riparius* (bottom).

Cilji raziskave so:

- opisati levitveni cikel in sintezo kutikule pri jamski mokrici ter rezultate primerjati z znanimi podatki o površinskih mokricah;
- opisati zgradbo in sestavo začasnih depozitov kalcija v levitvenem ciklu ter na podlagi pridobljenih rezultatov postaviti model nalaganja in resorpcije kalcijevih mineralov v založnih strukturah;
- opisati zgradbo in sestavo kalcijevih telesc na mikroskopskem nivoju in ugotoviti njihovo funkcijo;
- ugotoviti funkcijo epitelijev, povezanih z začasnimi depoziti kalcija, v sintezi in razgradnji mineraliziranih matriksov ter transportu kalcija ter primerjati te procese med preučevanima vrstama in z razpoložljivimi podatki o transportu kalcija pri drugih rakih.
- opisati zgradbo ščetinastih žleznih organov, jo primerjati z integumentalnimi žlezami drugih vrst rakov in preko strukturne analize sklepati na njihovo funkcijo.

V skladu s cilji raziskave smo postavili naslednja raziskovalna vprašanja:

- Ali se zgradba sekrecijskih epitelijev med izgradnjo nove kutikule in proces njene sinteze pri podzemeljskih in površinskih mokricah razlikujeta?
- Ali so pri preučevanih rakih prisotni začasni depoziti kalcija?
- Kakšni sta zgradba in sestava začasnih depozitov kalcija?
- Ali se arhitektura in sestava kalcijevih telesc v levitvenem ciklu pri izbranih vrstah rakov spreminjata?
- Ali se v levitvenem ciklu spreminja ultrastruktura epitelija kalcijevih telesc v povezavi z nalaganjem in resorpcijo mineralov?
- Ali se razlikujejo matriksi začasnih depozitov mineralov pri podzemeljskih in površinskih mokricah?
- Ali imajo žleze ščetinastih žleznih organov različnih rodov trihoniscidov podobno zgradbo?

2. ZNANSTVENA DELA

2.1 MIKROSKOPSKA ANATOMIJA TEGUMENTALNIH ŽLEZ SAMCEV IN Z NJIMI POVEZANIH KUTIKULARNIH STRUKTUR VRSTE *Titanethes albus* (CRUSTACEA: ISOPODA)

Microscopic anatomy of male tegumental glands and associated cuticular structures in *Titanethes albus* (Crustacea: Isopoda)

Miloš Vittori, Rok Kostanjšek, Nada Žnidaršič in Jasna Štrus

Arthropod Structure & Development, 2012, 41: 133-144

Žlezni organi samcev, za katere so značilne porozne površine ter ščetinaste kutikularne strukture so znane pri večih enakonožcih iz družine Trichoniscidae. Pri podzemeljski vrsti *Titanethes albus* so dorzalno na pleonu samcev prisotni parni tuberkli s številnimi ščetinicami in porami. Mikroskopsko anatomijo teh struktur smo analizirali s presevno in vrstično elektronsko mikroskopijo. Na tuberklih so prisotne raznolike epikutikularne strukture in številne senzile. Na pleonu smo ob dorzalnih tuberklih opazili tudi več dodatnih sekrecijskih površin. S svetlobno in presevno elektronsko mikroskopijo smo preučili tudi porazdelitev, arhitekturo in ultrastrukturo samčevih žlez vrste *T. albus*. V različnih delih pleona in v uropodih so prisotni trije različni tipi rozetastih žlez, značilnih za samce. Žleze z izvodili, ki se odpirajo na dorzalne tuberkle, vsebujejo zvezdaste osrednje celice. Ultrastruktura in histokemijske značilnosti samčevih žlez pri *T. albus* kažejo, da žleze izločajo peptide, ki morda opravljajo funkcijo kontaktnih feromonov.

2.2 LEVITEV IN TVORBA KUTIKULE PRI PODZEMELJSKEM TRIHONISCIDU VRSTE *Titanethes albus* (CRUSTACEA, ISOPODA)

Molting and cuticle deposition in the subterranean trichoniscid *Titanethes albus*
(Crustacea, Isopoda)

Miloš Vittori, Rok Kostanjšek, Nada Žnidaršič in Jasna Štrus

ZooKeys, 2012, 176: 23-38

Kopenski enakonožci so primerna skupina za študij sinteze kutikule ter dinamike kalcija, ker se pogosto levijo in so razvili strategije skladiščenja kalcija med levitvijo. Trenutno je na voljo le malo podatkov o levitvi pri skupini Synocheta in pri podzemeljskih enakonožcih. Preučili smo dinamiko levitve pri podzemeljskem trihoniscidu vrste *Titanethes albus* v laboratorijskih razmerah in mikroskopsko raziskali sternalne depozite CaCO₃ ter epitelij tergitev med levitvijo pri tej vrsti. V skladu z njenim počasnejšim metabolizmom je levitev vrste *T. albus* v laboratoriju okoli dva- do trikrat manj pogosta, kot bi pričakovali pri površinskih sorodnikih v podobnih razmerah. Živali so po levitvi posameznega dela telesa zavzele značilno držo in po posteriorni levitvi niso zaužile leva. Zgradba sternalnih depozitov kalcija in ultrastrukturne značilnosti epiderma med tvorbo kutikule so pri *T. albus* podobne tistim, ki so bile opisane pri predstavnikih družine Ligidae. Med nalaganjem eksokutikule oblikuje apikalna plazmalema celic epiderma prstaste izrastke in številne ugreznitve. V levitvenem prostoru osebkov v pozni predlevitveni fazi smo opazili dolge celične izrastke, obdane s snopi tubulov.

Molting and cuticle deposition in the subterranean trichoniscid *Titanethes albus* (Crustacea, Isopoda)

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Abstract

Terrestrial isopods are a suitable group for the study of cuticle synthesis and calcium dynamics because they molt frequently and have evolved means to store calcium during molt. Little data is currently available on molting in Synocheta and subterranean isopods. We studied the molting dynamics in the subterranean trichoniscid *Titanethes albus* under laboratory conditions and performed a microscopic investigation of sternal CaCO₃ deposits and the tergal epithelium during molt in this species. In accordance with its lower metabolic rate, molting in the laboratory is roughly 2–3 times less frequent in *T. albus* than would be expected for an epigeic isopod under similar conditions. Animals assumed characteristic postures following the molt of each body half and did not consume the posterior exuviae after posterior molt. The structure of sternal calcium deposits and the ultrastructural characteristics of the epidermis during cuticle formation in *T. albus* are similar to those described in representatives of Ligiidae. During the deposition of the exocuticle, the apical plasma membrane of epidermal cells forms finger-like extensions and numerous invaginations. In the ecdysial space of individuals in late premolt we observed cellular extensions surrounded by bundles of tubules.

Keywords

Cuticle ultrastructure, troglöbite, calcium storage

Introduction

Terrestrial isopods are known to molt frequently throughout their life cycle, making them particularly suitable for the study of cuticle synthesis and mineralization (Price and Holdich 1980). Furthermore, the onset of premolt can be easily determined in many terrestrial isopods due to the appearance of sternal calcium deposits (Zidar et al. 1998).

Isopods molt in two phases, first shedding the posterior and then the anterior half of the body. The boundary between the two halves is between pereionites 4 and 5. This pattern of biphasic molt is convenient and enables the simultaneous observation of the integument just prior to molt in the anterior half and just after molt in the posterior half of the same specimen.

Within Oniscidea, several studies have dealt with the ultrastructural aspects of cuticle deposition in Ligiidae (Glötzner and Ziegler 2000, Štrus and Blejec 2001) and in some members of the most terrestrial group, Crinocheta (Price and Holdich 1980, Compere 1990, Ziegler 1997). The ultrastructure of sternal CaCO_3 deposits has been analyzed in representatives of Ligiidae and several species of Crinocheta (Ziegler and Miller 1997). Their composition and formation have been studied in great detail in *Porcellio scaber* (reviewed in Ziegler et al. 2005). Data on cuticle synthesis in Synocheta are lacking, although there is some morphological information on calcium storage in this group (Verhoeff 1926, Ziegler 2003).

Caves are stable but nutrient poor habitats characterized by constant temperature corresponding to the average year temperature on the surface, permanent darkness and near-saturated relative humidity of air. Troglobitic animals have evolved specific adaptations to this environment, such as reduced pigmentation, thin cuticles, and lowered metabolism (Romero and Green 2005). *Titanethes albus* (C. Koch) is a large (about 1.5 cm in length) troglobitic representative of the family Trichoniscidae. The species inhabits wet limestone caves in the Dinaric Karst (Strouhal 1939) and is not exclusively terrestrial, as it is known to enter the water and can survive submerged for long periods (Sket 1986). The tergal cuticle of *T. albus* is thin compared to non-troglobitic isopods of similar size. It is also less mineralized and differs from the cuticles of non-troglobitic oniscids in its mineral composition, having a lower content of magnesium and calcite (Hild et al. 2009).

In our study, we observed the temporal dynamics of molt in a laboratory culture of *T. albus*. We provide an ultrastructural description of tergal cuticle deposition in this species and describe the characteristics of its sternal CaCO_3 deposits.

Methods

Laboratory culture and molt cycle observations

Specimens of *T. albus* from caves in central Slovenia were kept in the speleobiological laboratory at the Department of Biology, University of Ljubljana. The laboratory culture was maintained in a dark climate chamber at 11 ± 1 °C, the approximate average temperature of caves in central Slovenia. Animals were kept in glass containers with flowstone rocks, substrate from the sampling sites and spring water. Decaying wood and carrots were provided as food.

Individuals in culture were inspected for sternal deposits every month. Animals with sternal deposits were isolated into Petri dishes containing wet filter paper and observed daily. Every week, the sternal deposits were observed under a stereomicroscope

and their shape was drawn. After the first molt, specimens that were not fixed for microscopic examination were kept individually separated and were inspected weekly for the presence of sternal deposits in order to determine the onset of the following premolt.

Light microscopy and transmission electron microscopy

For ultrastructural observations, animals in premolt (determined by the presence of sternal deposits), intramolt (between the posterior and anterior molt), postmolt (1–2 days after the anterior molt), and intermolt were fixed. Individuals without sternal deposits that did not molt in the previous three weeks were considered to be in the intermolt stage.

Animals were dissected and isolated anterior tergites were fixed in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M cacodylate buffer (pH = 7.3) at 4 °C for at least a week. Specimens were postfixed with 1% OsO₄ for 1 hour, dehydrated in a graded ethanol series and embedded in Spurr's resin. Semithin (0.5 μm) sections were transferred to polylysine coated slides, stained with a mixture of Azur II and Methylene blue (Richardson et al. 1960) and imaged with an AxioImager Z.1 microscope (Zeiss) equipped with an HRc Axiocam camera. Thin (70 nm) sections were collected on copper grids, contrasted with uranyl acetate and lead citrate and examined with a CM 100 transmission electron microscope (FEI). Electron micrographs were recorded with a 792 BioScan camera (Gatan).

Scanning electron microscopy of sternal deposits

In preparation for scanning electron microscopy of sternal deposits, sternites of pereonites 1–4 with fully developed CaCO₃ deposits from premolt and intramolt specimens were removed, immersed in methanol and then air dried. When dry, the sternites were attached to aluminum holders and cleaved on an ultramicrotome with a glass knife. Samples were then sputter coated with platinum and imaged with a JSM-7500F field emission scanning electron microscope (JEOL).

Results

Duration of molt cycle

In the individuals studied, the median premolt duration (measured from the appearance of sternal deposits to the onset of molt) was 7 weeks (N=10). The shortest and longest premolt lasted 4 and 9 weeks, respectively. The median length of the period between the completion of molt and the second appearance of sternal deposits in non-ovigerous individuals was 11 weeks (N=9), with extreme values of 9 and 19 weeks. The only observed intermolt period of an ovigerous female lasted 34 weeks, with the release

of brood after 30 weeks. The anterior ecdysis followed 3 to 5 days after the posterior ecdysis. A diagrammatic representation of a typical molt cycle is provided in Fig. 1A. Some processes indicated on the diagram will be explained later in text.

After molting the posterior exuvium, the animals hold the newly molted body-half upwards so that it does not touch the substrate (Fig. 1B). The posterior three pairs of pereopods lie closely appressed against the ventral body surface and the entire body is supported by the anterior four pairs of pereopods. Animals maintain this posture for several hours, but they begin using the posterior pereopods before the onset of anterior molt. After the anterior molt, the anterior body-half is held upwards in a similar manner, with the body now supported by the posterior three pairs of pereopods (not shown).

In the laboratory, animals never consume their posterior exuviae during intramolt and no part of the shed exoskeleton is consumed directly upon the completion of molt. Exuviae (mostly of the posterior body-half), demonstrably belonging to *T. albus* due to the presence of gland-piliferous organs on the fourth pleonite, were also found on rocks in sampling localities (Fig. 1C), indicating that animals relinquish their old exoskeletons in nature as well.

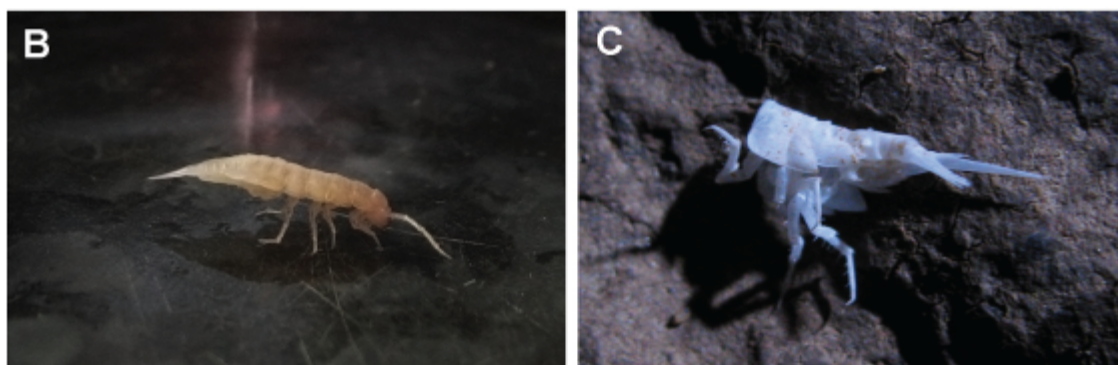
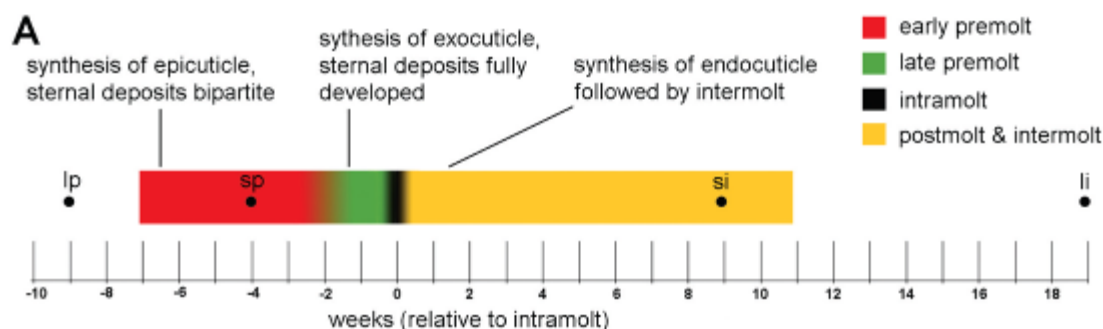


Figure 1. Molting in *Titanethes albus*. **A** a diagram of a typical molt cycle. The colored line shows the median observed durations of premolt and postmolt with intermolt. Different colors represent individual stages in the molt cycle. Key processes in each stage (early premolt, late premolt, and postmolt with intermolt) are indicated. Black dots indicate the onset of the longest and shortest observed premolt stages and the end of the longest and shortest observed intermolt stages **B** *T. albus* immediately after posterior molt. The posterior half of the body is held upwards while the body is supported solely by the anterior four pairs of pereopods **C** the posterior exuviae of *T. albus* on a rock in Viršnica Cave. **li** end of longest observed intermolt stage **lp** onset of longest observed premolt stage **si** end of shortest observed intermolt stage **sp** onset of shortest observed premolt stage.

Sternal CaCO₃ deposits

Like other oniscids, *T. albus* develops sternal CaCO₃ deposits in the ecdysial space of the anterior four sternites in premolt. Initially, the deposits are bipartite, with an anterior and a posterior part on each of the first four sternites of the pereion. The shape of sternal deposits in early premolt varies greatly between individuals. The anterior part of individual deposits is always larger and symmetrical (Fig. 2A), whereas the posterior part is smaller and irregular in shape. Sternal deposits in early premolt often display small round fenestrations (Fig. 2A). The location of these fenestrations on the deposits is highly variable. After their initial appearance, the shape of sternal deposits remains unaltered throughout most of the premolt stage. Towards the end of this stage, their shape changes rapidly, the two parts of each deposit fuse and the deposits assume a characteristic, uniform shape (Fig. 2B) in most individuals. Animals molt within a week after this change in the shape of the sternal deposits.

As revealed by scanning electron microscopy, fully formed sternal deposits of *T. albus* are composed of spherules, most of which measure 0.3 µm in diameter and vary in size between 0.1 µm and 1 µm (Fig. 2C). Deposits become progressively more compact from the sternal epidermis towards the old cuticle and spherules in the distal parts of deposits appear fused (Fig. 2D).

Cuticle deposition

Early premolt

The onset of apolysis is observable in the anterior tergites of animals in which the sternal deposits have just appeared. The epidermis and the old cuticle are in close proximity, but protrusions of the apical plasma membrane of epidermal cells with dense tips are already evident within the narrow ecdysial space (Fig. 3A). Later, the ecdysial space is wide and a fibrous and finely granular sheet is present in its distal part (Fig. 3B). The new epicuticle is initially synthesized as a thin electron dense layer over the short protrusions of the apical plasma membrane (Fig. 3C). Gaps in the epicuticle are visible in early stages of its synthesis, indicating that it is discontinuous in the initial stages of its deposition. Oblique sections through the apical epidermal surface suggest that the gaps are perforations of the epicuticle (Fig. 3D). Epicuticular protrusions (scales or hairs) begin to form around cell projections (Fig. 3E), but are initially flat and thinner than the corresponding epicuticular structures in intermolt. Epidermal cells possess a well developed rough endoplasmic reticulum (RER) (Fig. 3E).

Late premolt

In animals with fully formed sternal deposits, lamellae of the exocuticle are being deposited. During the synthesis of the distal dense layer (cf. Hild et al. 2009), the apical plasma membrane of epidermal cells forms finger-like extensions in addition to short

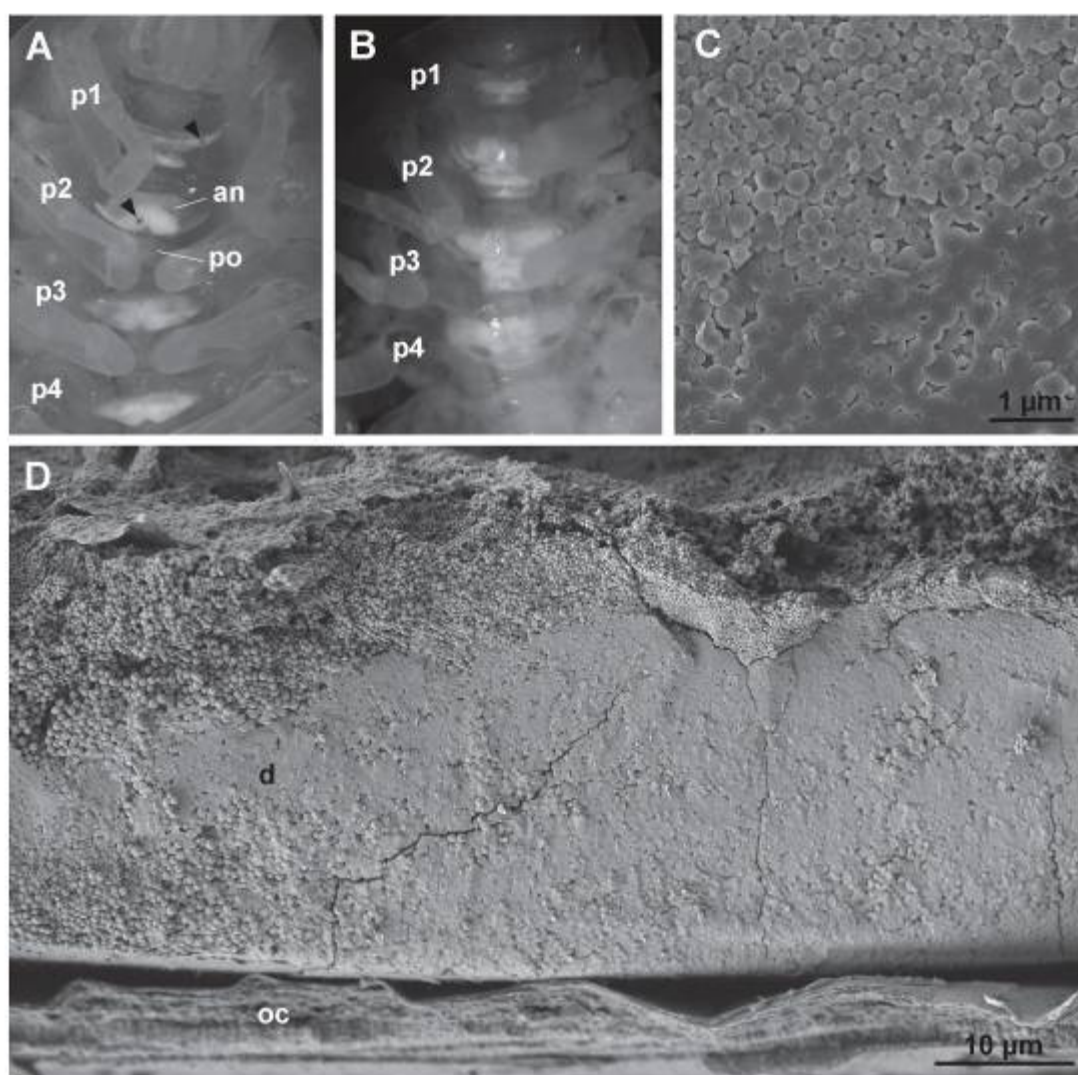


Figure 2. Sternal CaCO_3 deposits in *Titanethes albus*. **A** individual in early premolt with sternal deposits on anterior four pereionites. Deposits are bipartite with a larger anterior part (**an**) and a smaller posterior part (**po**). Round fenestrations (arrowheads) perforate the deposits. **B** individual in late premolt with fully developed sternal deposits. The anterior and posterior part on each segment are fused and the deposits have a uniform shape. **C** scanning electron micrograph of spherules forming the sternal deposits in late premolt. **D** scanning electron micrograph of a cleaved sternal deposit (**d**) in late premolt. Spherules are proximally more loosely arranged. **p1** pereionite 1, **p2** pereionite 2, **p3** pereionite 3, **p4** pereionite 4, **oc** old cuticle.

protrusions (Fig. 4A). The Golgi apparatus is well developed in addition to the RER (Fig. 4A). At this stage, the epicuticle and the structures it forms are already fully developed (Fig. 4A, B).

When the exocuticle consists of 3–5 lamellae, small electron dense vesicles appear in the apical cytoplasm of epidermal cells with well developed RER and numerous mitochondria (Fig. 4B). At this stage, the apical plasma membrane of epidermal cells forms numerous invaginations in addition to protrusions (Fig. 4C). Long cytoplasmic

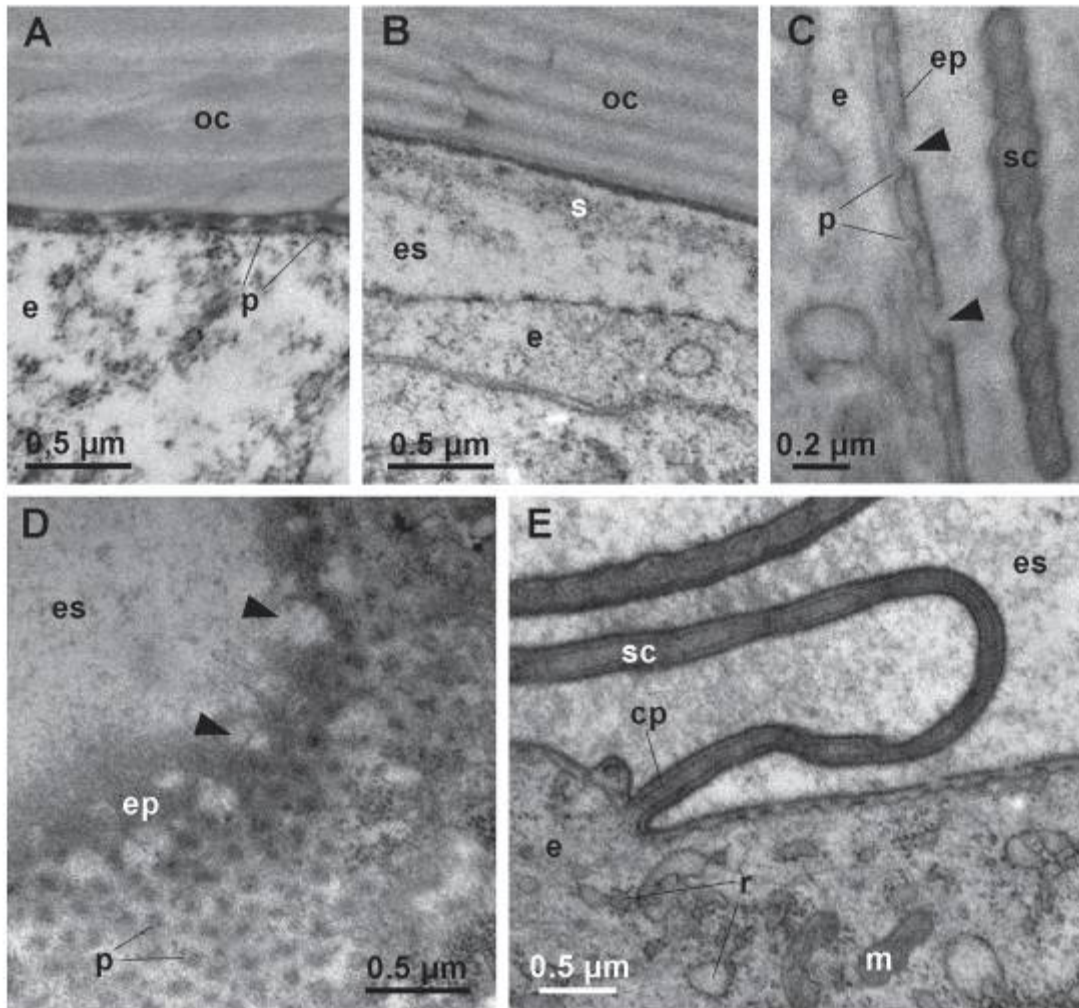


Figure 3. Ultrastructure of anterior tergites in early premolt. **A** apolysis. The apical surface of the epidermal cell (**e**) is detached from the old cuticle (**oc**), but the ecdysial space is narrow. The apical plasma membrane of epidermal cells forms short protrusions (**p**) with electron dense tips. **B** the ecdysial space in early premolt. A sheet of fibrous and granular material (**s**) is located in the distal part of the ecdysial space (**es**). **C** section through the apical surface of an epidermal cell (**e**) in early premolt. Gaps (arrowheads) are present in the newly formed epicuticle (**ep**) that is formed over short protrusions (**p**) of the apical plasma membrane. A developing epicuticular scale (**sc**) is visible. **D** oblique section through the apical surface of an epidermal cell in early premolt. Gaps (arrowheads) in the newly deposited epicuticle (**ep**) appear to be perforations. **E** the epidermis in early premolt. Epidermal cells (**e**) contain numerous mitochondria (**m**) and a well developed RER (**r**). Scales (**sc**) are forming around elongated projections of the apical plasma membrane (**cp**).

extensions reaching into pore canals and extending to the distal dense layer of the new exocuticle become evident (Fig. 4C).

In intramolt, as the anterior tergites are nearing molt, the new exocuticle approaches its final thickness (Fig. 4D). Epidermal cells maintain the characteristics of the late premolt stage with small electron dense vesicles in the apical cytoplasm and a well developed RER (Fig. 4D). Cytoplasmic extensions in pore canals are prominent (Fig. 4D, E) and the apical plasma membrane still forms numerous short protrusions with dense tips (Fig. 4E).

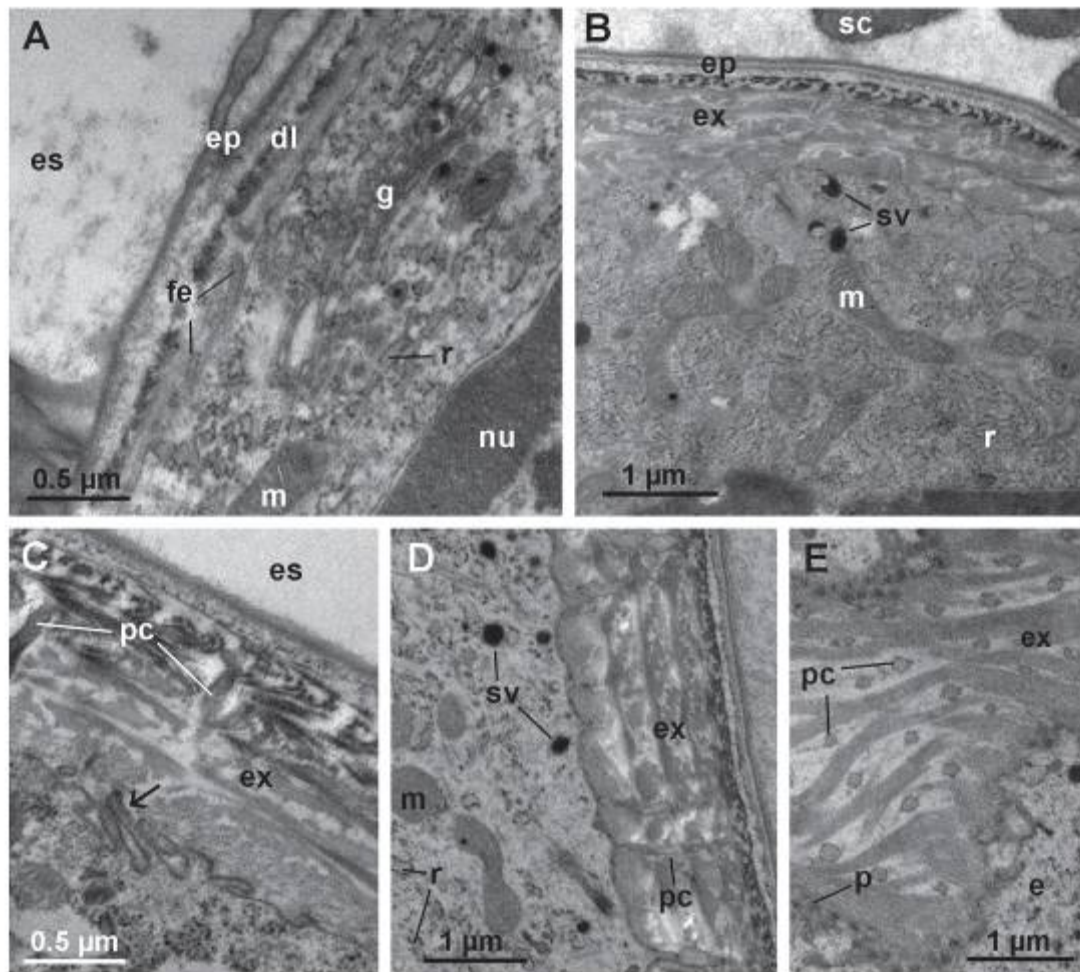


Figure 4. Ultrastructure of anterior tergites in late premolt and intramolt. **A** early stage of exocuticle formation. The distal dense layer (**dl**) is deposited. The apical plasma membrane of epidermal cells forms finger-like extensions (**fe**). The Golgi apparatus (**g**) is well developed. **B** anterior tergite in late premolt. Several lamellae of the new exocuticle (**ex**) are deposited. Epidermal cells contain a well developed RER (**r**), numerous mitochondria (**m**) and small electron dense vesicles (**sv**) in their apical cytoplasm. The epicuticle (**ep**) with scales (**sc**) is fully formed. **C** the apical plasma membrane of an epidermal cell in late premolt forms numerous invaginations (arrow). Cytoplasmic extensions reach into pore canals (**pc**). **D** anterior tergite in intramolt. The new exocuticle is almost fully deposited. The apical cytoplasm of epidermal cells contains electron dense vesicles (**sv**), numerous mitochondria (**m**) and a well developed RER (**r**). Long cytoplasmic extensions reach into pore canals (**pc**). **H** oblique section through the apical surface of an epidermal cell in intramolt. Pore canals (**pc**) in the new cuticle contain cytoplasmic extensions of epidermal cells. Numerous short protrusions (**p**) of the apical plasma membrane with dense tips are visible. **es** ecdysial space. **nu** nucleus.

During late premolt and intramolt stages, tubular bundles are present in the ecdysial space (Fig. 5A, B). The tubules, each measuring about 20 nm in diameter, protrude from the epicuticle in a regular arrangement and are covered by a diffuse electron dense material (Fig. 5B, C). Towards the old cuticle the regular arrangement of tubules is lost and the tubules disperse (Fig. 5B). At the center of each bundle of tubules there is a cellular extension containing parallel microtubules. The extension is enclosed in an elec-

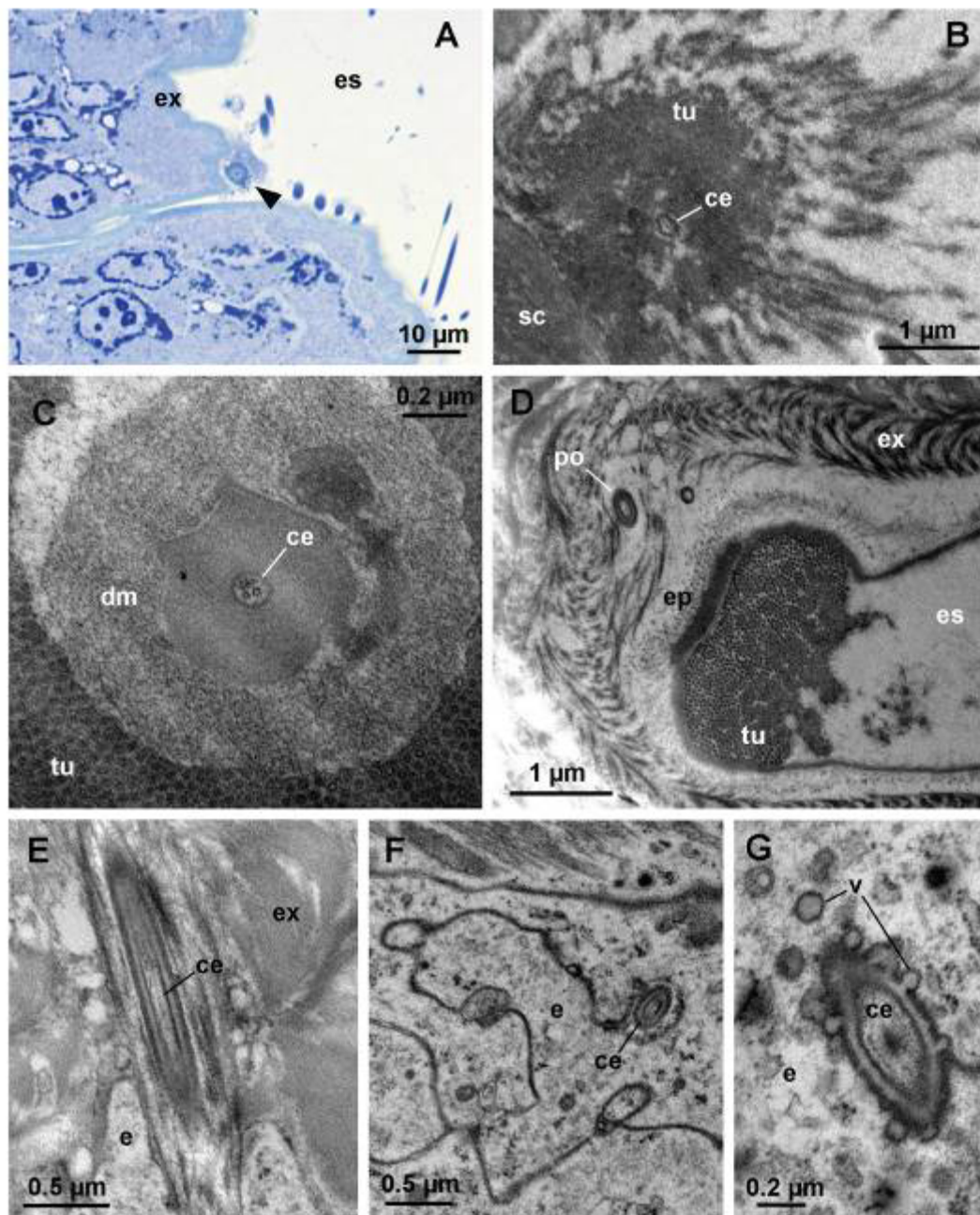


Figure 5. Cellular extensions and tubules in the ecdysial space. **A** oblique semithin section through the dorsal surface of an anterior tergite in intramolt. A bundle of tubules (arrowhead) on the surface of the new exocuticle (**ex**) is seen in cross-section. **B** electron micrograph of a bundle of tubules (**tu**). Proximally, the tubules are very densely arranged and they dissociate distally. A cellular extension (**ce**) within an electron dense sheath is located at the center of the bundle. **C** cross-section through a bundle of tubules. The cellular extension (**ce**) at the center of the bundle contains microtubules. Dense material (**dm**) surrounds the extension. **D** oblique section through the base of a bundle in intramolt. A pore (**po**) in the new cuticle is located beneath the bundle. Tubules (**tu**) protrude from the surface of the epicuticle (**ep**). **E** longitudinal section through a pore beneath a bundle of tubules. The pore contains a cellular extension (**ce**). **F** section through the epidermis beneath a bundle of tubules. A cellular extension (**ce**), enclosed in a sheath, is located in an invagination of an epidermal cell (**e**). **G** section through the cellular extension at the level of the epidermis. Vesicles (**v**) are present in the cytoplasm of the epidermal cell and are fused with the plasma membrane in proximity of the cellular extension. **es** ecdysial space, **sc** scale

tron dense sheath (Fig. 5C) and passes through the newly formed cuticle via a narrow pore (Fig. 5D, E). At the level of the epidermis, the cellular extension is enveloped by an epithelial cell (Fig. 5F). Small vesicles are present in the cytoplasm of the epidermal cell in the proximity of the extension. The membrane of some vesicles is continuous with the plasma membrane of the epithelial cell surrounding the extension (Fig. 5G).

A schematic representation of a tubular bundle surrounding a cellular extension and associated structures in late premolt is provided in Fig. 6.

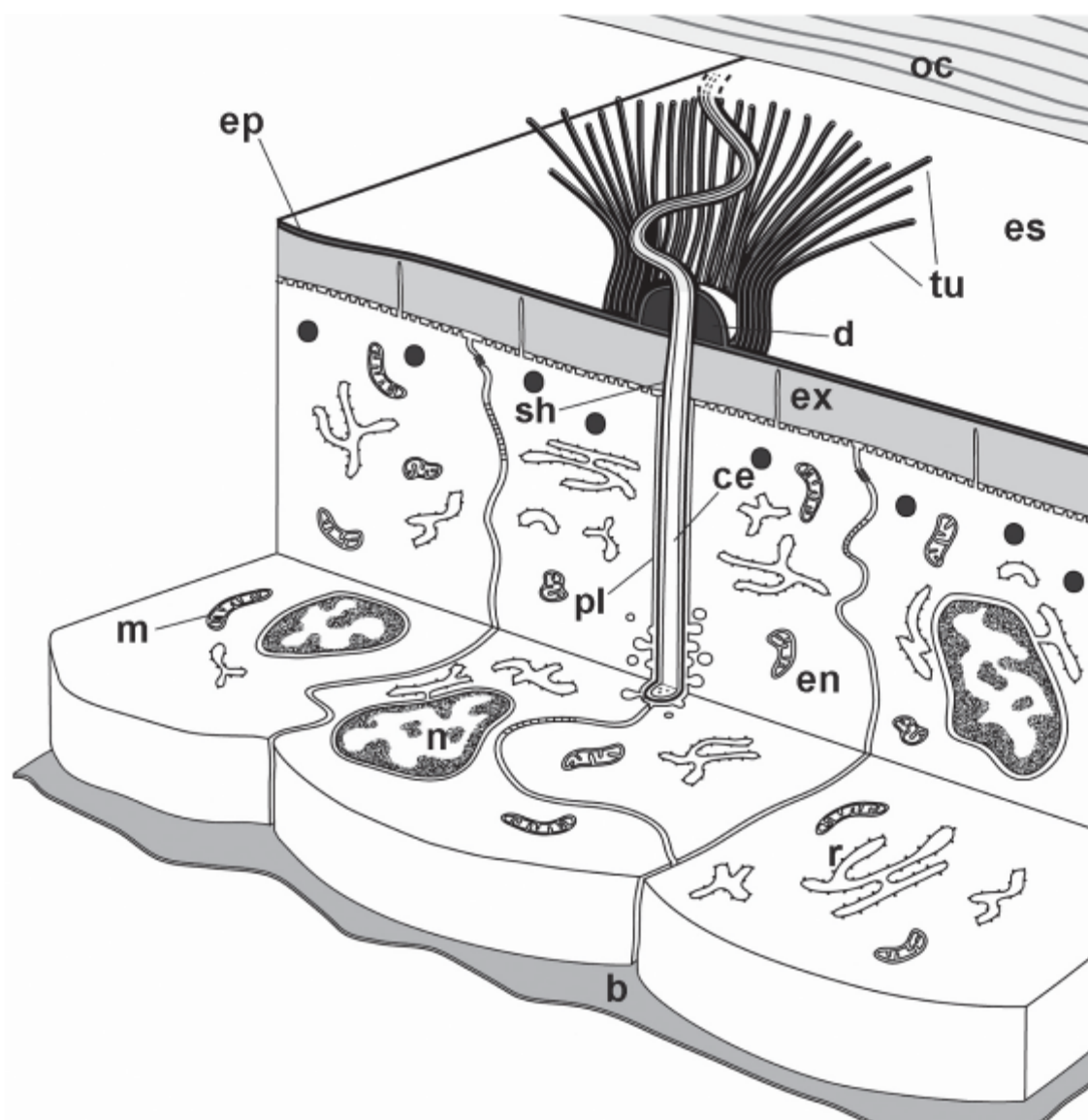


Figure 6. A schematic representation of cellular extensions associated with tubular bundles in the ecdysial space. A bundle of tubules (**tu**) surrounds a cellular extension (**ce**) that reaches into the ecdysial space (**es**). The cellular extension is enclosed in an electron dense sheath (**sh**). At the level of the epidermis, the cellular extension is surrounded by an enveloping epidermal cell (**en**). **b** basal lamina, **d** epicuticular thickening, **ep** new epicuticle, **ex** new exocuticle, **m** mitochondrion, **n** nucleus, **oc** old cuticle, **pl** plasma membrane of the enveloping epidermal cell, **r** RER.

Postmolt

During the first few days after anterior molt, rapid deposition of endocuticular lamellae takes place in anterior tergites (Fig. 7A, B). Small electron dense vesicles are no longer visible in the apical cytoplasm, but the RER remains well developed (Fig. 7B). The apical plasma membrane of epidermal cells still forms short protrusions with electron dense tips (Fig. 7B) and finger-like extensions may also be present (Fig. 7A). The cytoplasmic extensions in pore canals are less prominent than during intramolt and the pore canals appear electron lucent (Fig. 7A). After ecdysis, bundles of tubules remain present on the surface of the cuticle, but no cellular extensions can be observed

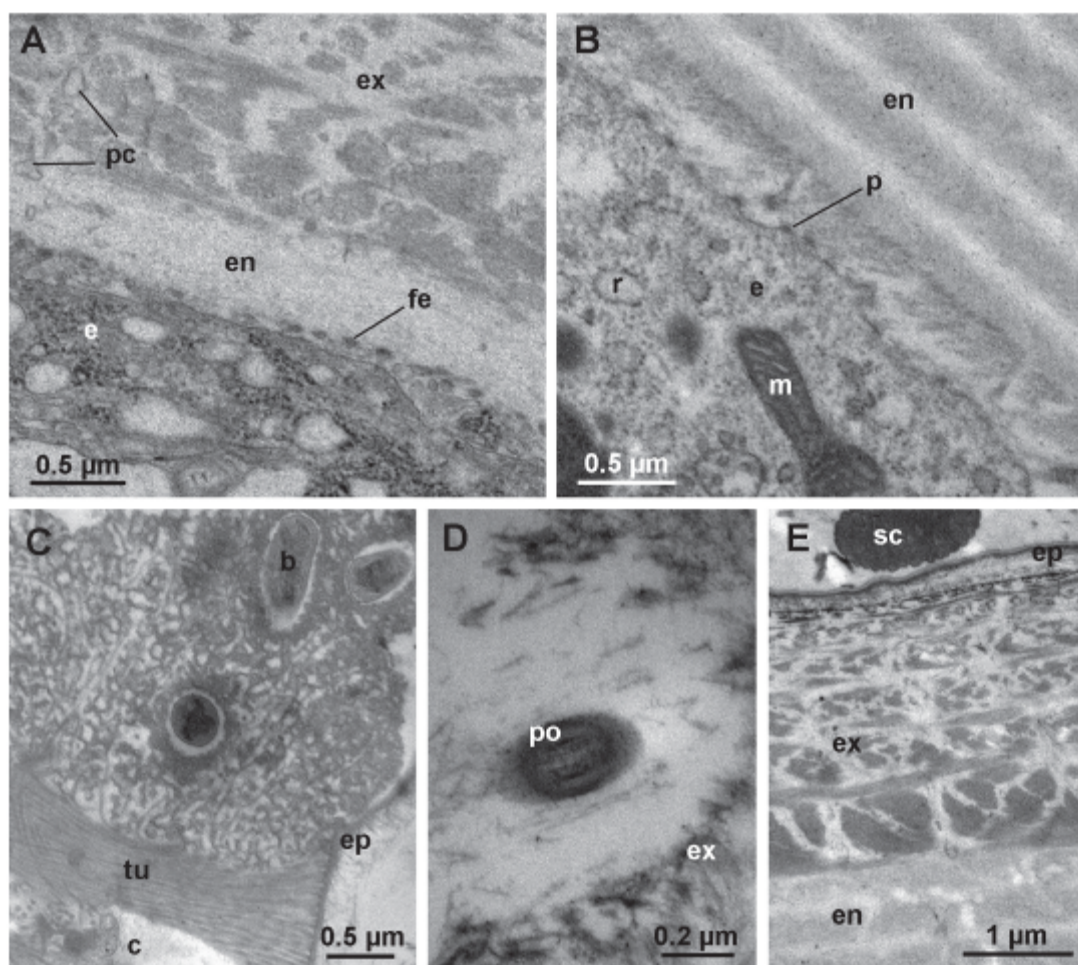


Figure 7. Ultrastructure of anterior tergites in postmolt. **A** anterior tergite shortly after molt. First lamellae of the endocuticle (**en**) are deposited proximally to the exocuticle (**ex**). Pore canals (**pc**) appear electron lucent. The apical plasma membrane of epidermal cells (**e**) forms finger-like extensions (**fe**). **B** apical region of an epidermal cell in postmolt. The epidermal cell (**e**) contains a well developed RER (**r**). The apical plasma membrane forms short protrusions (**p**) with dense tips. Several lamellae of the endocuticle (**en**) are deposited. **C** bundle of tubules (**tu**) protruding from the epicuticle (**ep**) of a tergite in postmolt. The center of the bundle (**c**) is electron lucent. **D** section through a pore (**po**) in the exocuticle beneath a bundle of tubules in postmolt. The lumen of the pore is electron dense. **E** The epicuticle (**ep**), exocuticle (**ex**) and endocuticle (**en**) in intermolt. **b** bacterium, **m** mitochondrion, **sc** scale.

in their proximity (Fig. 7C). The pores enabling the ensheathed cellular extensions at the centers of bundles to pass through the new cuticle in late premolt are observable in the cuticle in postmolt, but they appear very electron dense (Fig. 7D).

The fully synthesized tergal cuticle of an intermolt specimen of *T. albus* is presented in Figure 7E.

Discussion

The duration of the molt cycle as well as the length of individual stages within the cycle showed a high degree of variability in *T. albus* individuals, even under laboratory conditions. A typical molt cycle in *T. albus* is several times longer than in epigean isopods studied to date (Zidar et al. 1998, Štrus and Blejec 2001), but it is much shorter than that of some aquatic subterranean isopods, which molt every 9–18 months (Magniez 1975). It is difficult to compare data from studies of epigean oniscids with the results of our study, as most observations of molt cycles in terrestrial isopods were performed at room temperature while we maintained the *T. albus* culture at 11°C. By observing animals at different temperatures, Steel (1980) determined the value of Q_{10} for molting frequency in *Oniscus asellus*. On the basis of his study the expected molt cycle duration in *O. asellus* at 12 °C is approximately 8 weeks. The molt cycle in *T. albus* under laboratory conditions is thus about two times longer than would be expected in a similarly sized epigean species at a temperature close to 11 °C. This difference corresponds to different rates of respiratory metabolism between non-troglobitic isopods and *T. albus*. The expected rate of O_2 consumption of *O. asellus* at 10 °C, calculated from the previously measured metabolic rates (Phillipson and Watson 1965) and the Q_{10} for O_2 consumption in *O. asellus* (Nash 1979), would be around 0.1 ml O_2 g⁻¹h⁻¹, and would therefore be roughly three times higher than the measured rate of O_2 consumption in *T. albus* at 10 °C (Simčič et al. 2010). Rates of O_2 consumption at 10 °C for the amphibious isopod *Ligia italica* (Simčič et al. 2010) and the terrestrial species *Porcellio laevis* (Husain and Alikhan 1979) are similar to the value expected for *O. asellus*. Other troglobitic crustaceans are also known to have a lower metabolic rate compared to related non-troglobitic species, which is likely to be an adaptation to the nutrient-poor cave environment (Hervant et al. 1997).

The specific postures assumed by *T. albus* after the posterior and anterior molt closely resemble those described in the epigean isopod *Armadillo officinalis* (Verhoeff 1940). The lifting of the newly molted body-half from the substrate thus appears to be widespread in Oniscidea, although it has not been reported in all species studied.

Sternal deposits of *T. albus*, consisting entirely of spherules, resemble the sternal deposits described in members of the family Ligiidae (Ziegler and Miller 1997) and *T. albus* is the first species outside Ligiidae known to form deposits of this type. There is little data on sternal deposits in Synocheta, but it has been suggested that some representatives of the group employ three-layered deposits (Ziegler 2003). If this is the case, the absence of a proximal homogenous layer in the sternal deposits of *T. albus* might represent a second-

ary reduction in the complexity of sternal deposits as an adaptation to the subterranean environment or to the amphibious mode of life of this species. This is further supported by the fact that three-layered deposits occur in Tylidae (Ziegler 2003), which is most likely the sister group of all other oniscids excluding Ligiidae (Schmidt 2008).

Consumption of the shed cuticle after ecdysis occurs in other crustaceans (Greenway 1985) and other arthropods, such as insects (Mira 2000). It is known that other terrestrial isopods also consume their exuviae after molting each body-half (Messner 1965, Ziegler et al. 2007). In contrast, *T. albus* does not ingest the posterior exuviae. Cuticle consumption as means of obtaining calcium required for the mineralization of the anterior exoskeleton is likely less crucial for the molting *T. albus*, as this species possesses very large internal calcium stores in the posterior body-half which can be utilized for cuticular mineralization after molt (personal observation). Internal calcium stores are also known to be present in some other trichoniscids (Verhoeff 1926, Ziegler 2003). It has been reported that the ligiids *Ligia hawaiiensis* (Ziegler et al. 2007) and *Ligia italica* (Štrus and Blejec 2001) also do not ingest the exuviae of at least one body-half. It was shown that *Ligia hawaiiensis* nevertheless retains a very high percentage of body calcium during molt which may relate to its lower body calcium content when compared to fully terrestrial isopods (Ziegler et al. 2007). Since *T. albus* also has a weakly mineralized exoskeleton and lives in a moist limestone environment, it probably has a lesser need for cuticle consumption than species that must shift greater amounts of calcium to their exoskeletons over a shorter period of time without relying on environmental calcium sources.

The ultrastructural characteristics of *T. albus* epidermal cells during cuticle synthesis, such as short protrusions of the apical plasma membrane, a well developed RER and abundant mitochondria throughout cuticle deposition as well as the presence of small, dense vesicles in the apical cytoplasm during exocuticle deposition are generally similar to those described in other oniscids (Price and Holdich 1980, Ziegler 1997, Štrus and Blejec 2001). Similar epithelial features are also present during molt in other crustacean groups (Koulis and Klepal 1981) and some aspects of epithelial ultrastructure during cuticle synthesis, for example the short protrusions of the apical plasma membrane, have also been found in insects (Locke 1961, 2001). In *T. albus*, the apical plasma membrane of epidermal cells appears highly structured during deposition of the distal lamellae of the exocuticle. Finger-like extensions and membrane invaginations may be involved in the synthesis of the exocuticle, but they might also function in intensive transport processes between the ecdysial space and the haemolymph. In *T. albus*, the epicuticle in late premolt (Fig. 3F) appears very similar to the intermolt epicuticle (Fig. 5E), indicating that there are little or no postecdysial modifications of this cuticular sublayer. This is not surprising since the epicuticular waxy layer which is modified after molt in *O. asellus* (Compere 1990) is absent in the epicuticle of *T. albus* (Hild et al. 2009). Also, the exocuticle in *T. albus* maintains its lamellar appearance after ecdysis and the premolt exocuticle greatly resembles the intermolt exocuticle (compare Figs 3G and 5E). The exocuticle in this species is therefore not deformed by the deposition of the endocuticle, as reported in *O. asellus* (Price and Holdich 1980).

The tubular structures reaching into the ecdysial space from the epicuticle in late premolt appear identical to those known from *Ligidium hypnorum* (Glötzner and Ziegler 2000) and *Ligia exotica* (Štrus et al. 2003). Tubules extending into the ecdysial space from the surface of the epicuticle therefore occur outside Ligiidae as well. In *T. albus*, they are very pronounced and associate with cellular extensions reaching through the newly formed cuticle into the ecdysial space. It has been suggested that tubules within the ecdysial space of isopods may function in water retention (Glötzner and Ziegler 2000). In the case of *T. albus*, their function is probably linked to cellular extensions reaching into the ecdysial space. The ensheathed, microtubule containing cellular extensions at the centers of tubular bundles resemble dendrites innervating isopod sensilla (cf. Crouau 1994), but their function remains to be established. The apparent absence of cellular extensions above the level of the new cuticle in proximity of the tubules in postmolt indicates that these are transient projections that degenerate after molt. The small vesicles associated with the cellular extensions in the epidermis suggest that intensive resorption and/or secretion of material may take place around the cell extensions at the level of the epidermis.

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2.3 KALCIJEVA TELESKA PRI VRSTI *Titanethes albus* (CRUSTACEA: ISOPODA): STRUKTURNA DINAMIKA V POVEZAVI Z LEVITVIJO IN S KALCIFICIRANIM MATRIKSOM POVEZANE BAKTERIJE

Calcium bodies of *Titanethes albus* (Crustacea: Isopoda): molt-related structural dynamics and calcified matrix-associated bacteria

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Raki izgrajujejo raznolike depozite kalcija, v katerih shranjujejo kalcij, potreben za mineralizacijo njihovih eksoskeletov. O kalcijevih telescih, organih, ki vsebujejo veliko kalcija, so poročali pri nekaterih rakah enakonožcih, vendar še niso bila podrobno preučena. Analizirali smo arhitekturo teh organov med levitvenim ciklom pri enakonožcu *Titanethes albus*. Posamezni organi so epiteljske vrečke, ki vsebujejo material, urejen v koncentrične plasti, ločene s tankimi laminami. Kot sta pokazali elektronska mikroskopija in fluorescentna hibridizacija *in situ*, so v kalcijevih telescih prisotne številne bakterije. Ne glede na fazo levitvenega cikla so v osrednjih delih kalcijevih telesc prisotni kristalni skupki. Energijsko-disperzijska rentgenska spektrometrija skupkov je pokazala, da so sestavljeni pretežno iz kalcija ter fosforja in elektronska difrakcija je nakazala prisotnost hidroksiapatita. Pri osebkih med levitvijo se med ovojnico in najbolj zunanjo lamino kalcijevih telesc oblikuje steklast sloj mineraliziranega matriksa. Ta sloj sestavlja amorfen kalcijev mineral, ki vsebuje manj fosforja kot osrednji skupki in se po levitvi resorbira. Ker so spremembe mineraliziranega matriksa usklajene z levitvenim ciklom, kalcijeva telesa verjetno opravljajo funkcijo založnega organa, ki dopolnjuje sternalne depozite kot vir kalcija za mineralizacijo eksoskeleta. Kaže, da bakterije, povezane z mineraliziranim matriksom kalcijevih telesc, sodelujejo v dinamiki kalcija.

2. 4 MINERALIZACIJA V KALCIJEVIH TELESCIH Z BAKTERIJAMI IN BREZ NJIH PRI RAKU *Hyloniscus riparius* (ISOPODA: ONISCIDEA)

Mineral deposition in bacteria-filled and bacteria-free calcium bodies in the crustacean
Hyloniscus riparius (Isopoda: Oniscidea)

Miloš Vittori, Alenka Rozman, Jože Grdadolnik, Urban Novak in Jasna Štrus

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Kalcijeva telesca rakov so epitelijske vrečke, ki vsebujejo mineraliziran matriks. Cilji te študije so bili raziskati mikroskopsko anatomijo kalcijevih telesc pri kopenskem enakonožcu *Hyloniscus riparius*, ugotoviti ali v njih prihaja do sprememb, povezanih z levitvijo, ter analizirati kemijsko sestavo mineraliziranih matriksov v njih. Izvedli smo 3D rekonstrukcijo kalcijevih telesc na podlagi serijskih parafinskih rezin ter analizirali njihovo strukturo s svetlobno in elektronsko mikroskopijo. Poleg tega smo analizirali sestavo njihovih mineraliziranih matriksov z mikro-ramansko spektroskopijo. Pri *H. riparius* sta prisotna dva para teh organov. Zadnji par je napolnjen z bakterijami, medtem ko jih v sprednjem paru ni. Pri živalih, ki se ne levijo, vsebujejo kalcijeva telesca z bakterijami kristale apatita, kalcijeva telesca brez bakterij pa mineralne skupke, ki vsebujejo CaCO₃ in malo organskega matriksa. Med pripravo na levitev se v obeh parih kalcijevih telesc naloži dodaten sloj matriksa. Kalcijeva telesca, napolnjena z bakterijami, vsebujejo kalcijev karbonat in kalcijev fosfat, medtem ko je v kalcijevih telescih brez bakterij prisoten zgolj kalcijev karbonat. Po levitvi se povsem resorbirajo vse mineralne komponente v kalcijevih telescih brez bakterij ter dodatni sloj matriksa v kalcijevih telescih z bakterijami. Med resorpcijo kalcija je apikalna površina epitelijskega kalcijevih telesc globoko nagubana in v prostorih med epitelijskimi celicami so prisotne elektronsko goste granule. Naši rezultati kažejo, da je prisotnost bakterij morda povezana z mineralizacijo kalcijevega fosfata. Kalcijeva telesca verjetno služijo kot vir kalcija in potencialno fosfata za mineralizacijo nove kutikule po levitvi. Za razliko od drugih kopenskih enakonožcev *H. riparius* ne tvori sternalnih depozitov CaCO₃ in kalcijeva telesca brez bakterij jih pri tej vrsti morda funkcionalno nadomeščajo.

Mineral Deposition in Bacteria-Filled and Bacteria-Free Calcium Bodies in the Crustacean *Hyloniscus riparius* (Isopoda: Oniscidea)

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Abstract

Crustacean calcium bodies are epithelial sacs which contain a mineralized matrix. The objectives of this study were to describe the microscopic anatomy of calcium bodies in the terrestrial isopod *Hyloniscus riparius* and to establish whether they undergo molt-related structural changes. We performed 3D reconstruction of the calcium bodies from paraffin sections and analyzed their structure with light and electron microscopy. In addition, we analyzed the chemical composition of their mineralized matrices with micro-Raman spectroscopy. Two pairs of these organs are present in *H. riparius*. One pair is filled with bacteria while the other pair is not. In non-molting animals, the bacteria-filled calcium bodies contain apatite crystals and the bacteria-free calcium bodies enclose CaCO₃-containing concretions with little organic matrix. During preparation for molt, an additional matrix layer is deposited in both pairs of calcium bodies. In the bacteria-filled calcium bodies it contains a mixture of calcium carbonate and calcium phosphate, whereas only calcium carbonate is present in bacteria-free calcium bodies. After ecdysis, all mineral components in bacteria-free calcium bodies and the additional matrix layer in bacteria-filled calcium bodies are completely resorbed. During calcium resorption, the apical surface of the calcium body epithelium is deeply folded and electron dense granules are present in spaces between epithelial cells. Our results indicate that the presence of bacteria might be linked to calcium phosphate mineralization. Calcium bodies likely provide a source of calcium and potentially phosphate for the mineralization of the new cuticle after molt. Unlike other terrestrial isopods, *H. riparius* does not form sternal CaCO₃ deposits and the bacteria-free calcium bodies might functionally replace them in this species.

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Introduction

The calcification of the rigid crustacean exoskeleton requires an abundant and readily available source of calcium ions after each molt. As a response, different groups of crustaceans have evolved various transient calcium deposits to retain some of the calcium necessary for cuticular calcification after molt [1,2]. A general feature of terrestrial isopod crustaceans (Oniscidea) are sternal CaCO₃ deposits, which form before ecdysis in the ecdysial space (the space between the detached old cuticle and the underlying epidermis) of anterior sternites [3]. The process of molt in isopods is biphasic. During the posterior ecdysis, animals shed the cuticle of body segments posterior to pereonite 4. In the anterior ecdysis that follows, the body segments anterior to pereonite 5 are molted. Sternal CaCO₃ deposits are resorbed during the intramolt stage, the short period between the two partial ecdyses, and the mineral ions stored within them are utilized for the mineralization of the new exoskeleton [4].

Additionally, calcium deposits within specialized organs, the calcium bodies, have been reported in some representatives of the terrestrial isopod family Trichoniscidae [5,6]. The microscopic anatomy of these organs and the changes they undergo during the molt cycle have thus far only been studied in the subterranean

trichoniscid *Titanethes albus* [7]. In this species it was found that the calcium bodies are epithelial sacs filled with a bacteria-containing matrix mineralized with apatite. An additional glassy layer of mineralized matrix containing amorphous calcium minerals is formed in the calcium bodies of *T. albus* before molt and is degraded after it, suggesting that these organs have a role in calcium dynamics during molt. Whether the calcium bodies of other trichoniscids also harbor bacteria and what possible function these bacteria might perform is currently unknown.

In the trichoniscid *Hyloniscus riparius* (Koch), two pairs of calcium bodies, both located in pereonite 7, were described by Méhely [6]. Later, Ziegler [8] reported a single pair of calcium-containing epithelial sacs in this species, with several separate mineral concretions present within each sac. Elemental analysis demonstrated large amounts of calcium in the concretions with phosphorus appearing in the posterior concretions only [8]. In this study, we determined the exact number, position and structure of calcium bodies using serial sectioning and 3D reconstruction of the organs. Contrary to the previous reports, our results demonstrate that two pairs of calcium bodies, each being a separate epithelial compartment, are present in *H. riparius*. Furthermore, we found that one pair of calcium bodies contains bacteria while the other pair does not.

Information on possible changes of the calcium body epithelium ultrastructure and the mineralization of calcium body matrix during the molt cycle in *H. riparius* is lacking. Also, the chemical composition of the material within the calcium bodies of *H. riparius* has not been determined so far. In order to establish whether or not changes occur in the mineralized matrix and the epithelium of calcium bodies during the molt cycle, we analyzed their structure in different molt cycle stages with a combination of microscopic techniques. In addition, we determined the composition of the mineralized matrices within these organs with micro-Raman spectroscopy.

Our results indicate that calcium bodies are involved in calcium dynamics during molt. In *H. riparius*, calcium bodies harboring bacteria as well as those lacking them are present within the same organism. The comparison of both types of calcium bodies can provide insights into which features of their epithelia and mineralized matrices are possibly linked to the presence of bacteria and it may indicate the possible role of bacteria within calcium bodies.

Materials and Methods

Animal Culture

Specimens of *H. riparius* were sampled near Cerknica and Ormož in Slovenia. Animals were kept in glass containers within a climate chamber at 21°C with a 14 h photoperiod. They were fed with mixed leaf litter from the sampling localities. For determination of molt cycle stages, individuals were separated into plastic containers with wet filter paper and observed daily. Specimens in the 2–4 mm body length range were used in our analyses. No specific permits were required for field sampling.

Examined Stages of the Molt Cycle

We examined animals in five different stages of the molt cycle. Specimens with no sign of apolysis and in which ecdysis was not previously observed were considered to be in the intermolt stage. During this stage the exoskeleton is fully formed and the integumental epithelium does not synthesize cuticle. The absence of new cuticle synthesis was chosen as the criterion for intermolt stage. To rule out possible confusion with premolt stage specimens, the lack of new cuticle synthesis was confirmed microscopically on tissue sections of the posterior body-half in all specimens examined.

The premolt stage is the period from the apolysis (the detachment of the old cuticle from the epidermis) to the beginning of ecdysis [9] and in most terrestrial isopods the onset of premolt stage is easily recognized by the formation of sternal CaCO_3 deposits [10]. Since we established that sternal deposits are not formed in *H. riparius* (see Results), we identified premolt specimens by the presence of a transparent posterior edge on the pereon epimeres. In all such specimens, we additionally confirmed the presence of an ecdysial gap and the synthesis of the new cuticle, which were chosen as criteria for the premolt stage, on tissue sections of the posterior body-half.

Individuals in intramolt stage (between the posterior and anterior ecdyses) and postmolt stage (after the anterior ecdysis) were recognized by direct observation of the posterior ecdysis and the anterior ecdysis, respectively. Specimens were studied at two points in the postmolt stage: within a day after anterior ecdysis and two days after anterior ecdysis.

Histology

For serial paraffin sections and 3D reconstruction of calcium bodies, *H. riparius* specimens were anesthetized with diethyl ether

and fixed overnight in 4% aqueous formaldehyde at room temperature. The fixative was then washed and whole specimens were decalcified in 10% ethylenediaminetetraacetic acid (EDTA), dehydrated in an ethanol series and embedded in paraffin. Tissue sections (8 μm) were placed on glass slides, deparaffinized with xylene, rehydrated in an ethanol series, stained with hematoxylin and eosin and covered in Pertex (Medite). The shape of the calcium bodies' outer surface was reconstructed in the program Reconstruct [11] by manually tracing the calcium body epithelium on images of sequential sections. For examination of the mineralized matrices within calcium bodies in different molt cycle stages, whole specimens were fixed in 96% ethanol to prevent the dissolution of mineral components. Sections were prepared as described above and stained with eosin. Light micrographs were captured on an AxioImager Z.1 microscope (Zeiss) with the HRC AxioCam camera.

Semithin Sections and Transmission Electron Microscopy (TEM)

For transmission electron microscopy, pereons of *H. riparius* in different molt cycle stages were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer, pH = 7.3 at 4°C for several days. After washing of the fixative with 0.1 M cacodylate buffer, tissues were postfixed with 1% aqueous OsO_4 for 1 h, dehydrated in an ethanol series and embedded in Spurr's resin (SPI). Semithin sections (0.5 μm) were transferred to glass slides, stained with Azur II and Methylene blue [12] and imaged as described for the paraffin sections. Thin sections (70 nm) were collected on formvar-coated copper grids, contrasted with uranyl acetate and lead citrate and observed with a CM 100 transmission electron microscope (FEI). The preparation procedures for TEM generally resulted in the loss of calcium minerals from tissues.

Scanning Electron Microscopy (SEM)

For scanning electron microscopy, isolated calcium bodies or pereon halves were immersed in methanol for several hours and air dried in order to preserve mineral components. Alternatively, pereon halves of some specimens were fixed as for TEM, postfixed with 1% OsO_4 for 2h, dehydrated in an ethanol series followed by two changes of pure acetone and air-dried in Hexamethyldisilazane (HMDS) for the preservation of soft tissues. Dry samples were attached to brass holders with silver paint and fractured to expose the interior. Specimens were coated with a 14 nm thick layer of platinum using a BAL-TEC SCD 050 sputter coater and observed with a JEOL JSM-7500F field-emission scanning electron microscope.

Micro-Raman Spectroscopy

For micro-Raman spectroscopy, specimens were decapitated and dissected in absolute ethanol or methanol. Individual calcium bodies were isolated, air dried, placed on glass slides and covered with cover slips. For comparison, sternal deposits of *Titanethes albus* (Koch) were prepared the same way.

Raman spectra were recorded on a Bruker Ramascope III by application of laser excitation at 1064 nm. The laser radiation was transmitted to the microscope with an optical fiber and focused on the sample with a long working distance objective at $\times 40$ magnification. The Raman scattered light was collected with the same objective and detected with a liquid-nitrogen cooled Ge detector. Spectra were averaged from $\sim 19,000$ interferograms and apodised with a Happ-Genzel function. All spectra were recorded in the spectral range between 50 and 3600 cm^{-1} with the nominal resolution of 4 cm^{-1} . Laser power at the sample was

estimated to be approximately 100 mW. Reducing the laser power did not result in significant changes in the Raman spectra.

Results

1. Molting in *H. riparius*

As in other isopods, the molt in *H. riparius* is biphasic (Fig. 1 A) with the anterior ecdysis following the posterior ecdysis in less than a day. Animals that we observed never consumed the posterior exuviae, but often consumed the anterior exuviae after the anterior ecdysis.

Although animals in all molt cycle stages were carefully examined, no sternal deposits were ever observed in premolt and intramolt specimens (Fig. S1). Furthermore, the anterior sternal epithelium in intramolt stage lacks the ultrastructural characteristics reported in other terrestrial isopods during the resorption of sternal deposits (Fig. S1), such as a basolateral intercellular network and invaginations of the apical plasma membrane [13]. Thus, sternal CaCO_3 deposits are apparently entirely absent in *H. riparius*.

2. Number, Position and Structure of Calcium Bodies

As demonstrated by serial sectioning and 3D reconstruction from paraffin sections, two pairs of calcium bodies are present in the pereon of *H. riparius* (Figs. 1 B–D). The larger anterior pair extends from pereonite 4 to the middle of pereonite 6 and the smaller posterior pair from pereonite 6 to pleonite 1. Within pereonite 6, both pairs overlap as the anterior calcium bodies extend dorsally over the posterior ones; however, we established that there is no luminal connection between the anterior and posterior calcium bodies. Each calcium body is an epithelial sac consisting of a longitudinally oriented medial tube with a smaller lateral extension (Fig. 1 B). The organs are positioned ventrally in

the body, close to the hindgut and the hepatopancreatic tubes and the basal laminae of anterior calcium bodies and hepatopancreas are closely apposed. The anterior and posterior calcium bodies differ markedly in their contents; therefore, each pair will be considered separately.

Each posterior calcium body consists of a single-layered epithelium enclosing layered material (Fig. 2 A). As is evident from TEM and SEM observations, the posterior calcium bodies harbor large numbers of rod-shaped bacteria approximately $0.8 \mu\text{m}$ in length and $0.4 \mu\text{m}$ in diameter (Figs. 2 B, C). Dense layers of crystals approximately 100 nm in size are present among the bacteria (Figs. 2 D, E). Crystals are often associated with thin laminae, resulting in a layered appearance of the mineralized matrix (Fig. 2 D).

The posterior calcium body epithelium is lined apically by a 45 nm thick extracellular envelope, which encloses the material in the lumen (Fig. 3 A). The envelope consists of a thinner electron lucent layer facing the lumen and a thicker electron dense layer in contact with the epithelium (Fig. 3 B). Additional thin laminae are often present in the central area of the calcium body lumen. The cytoplasm of epithelial cells with intensely folded apical membranes contains numerous mitochondria and abundant electron dense particles, which form rosettes and structurally resemble glycogen (Fig. 3 C). In addition, electron dense vesicles (Fig. 3 B) and large organelles filled with membranous structures (possibly autophagic vacuoles) are common in the cytoplasm of epithelial cells (Fig. 3 D). Cells interconnect by subapical adherens junctions and by more basally positioned septate junctions (Fig. 3 E).

As in posterior calcium bodies, the wall of the anterior calcium bodies is formed by a single-layered epithelium (Fig. 4 A), but no bacteria were ever observed in their lumen. Instead, the anterior calcium bodies in intermolt stage contain glassy concretions (Figs. 4 B, C). These concretions consist of a continuous layer of mineralized

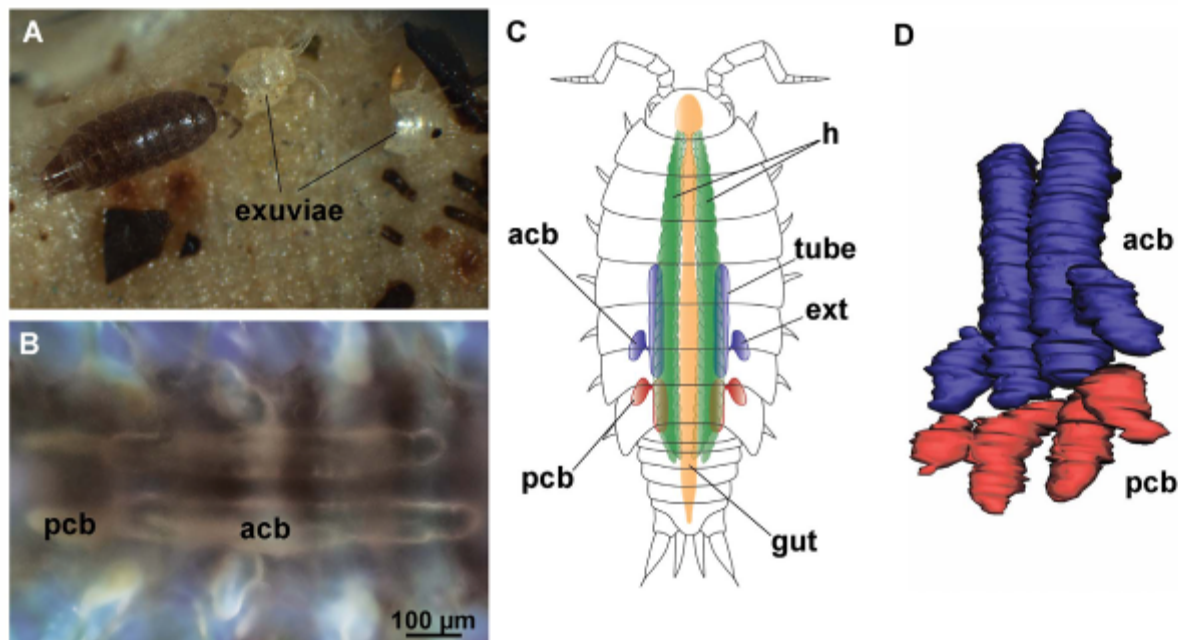


Figure 1. Molting and calcium bodies in *Hyloniscus riparius*. (A) An individual shortly after anterior ecdysis. Exuviae of both body-halves are visible in the proximity. (B) Dark-field image of the posterior (pcb) and anterior (acb) calcium bodies *in situ*. (C) An illustration of the position of the digestive system and the calcium bodies in the body. Each calcium body consists of a medial tube and a lateral extension (ext). (D) 3D reconstruction of calcium bodies from serial paraffin sections, dorsolateral view. h – hepatopancreas.
doi:10.1371/journal.pone.0058968.g001

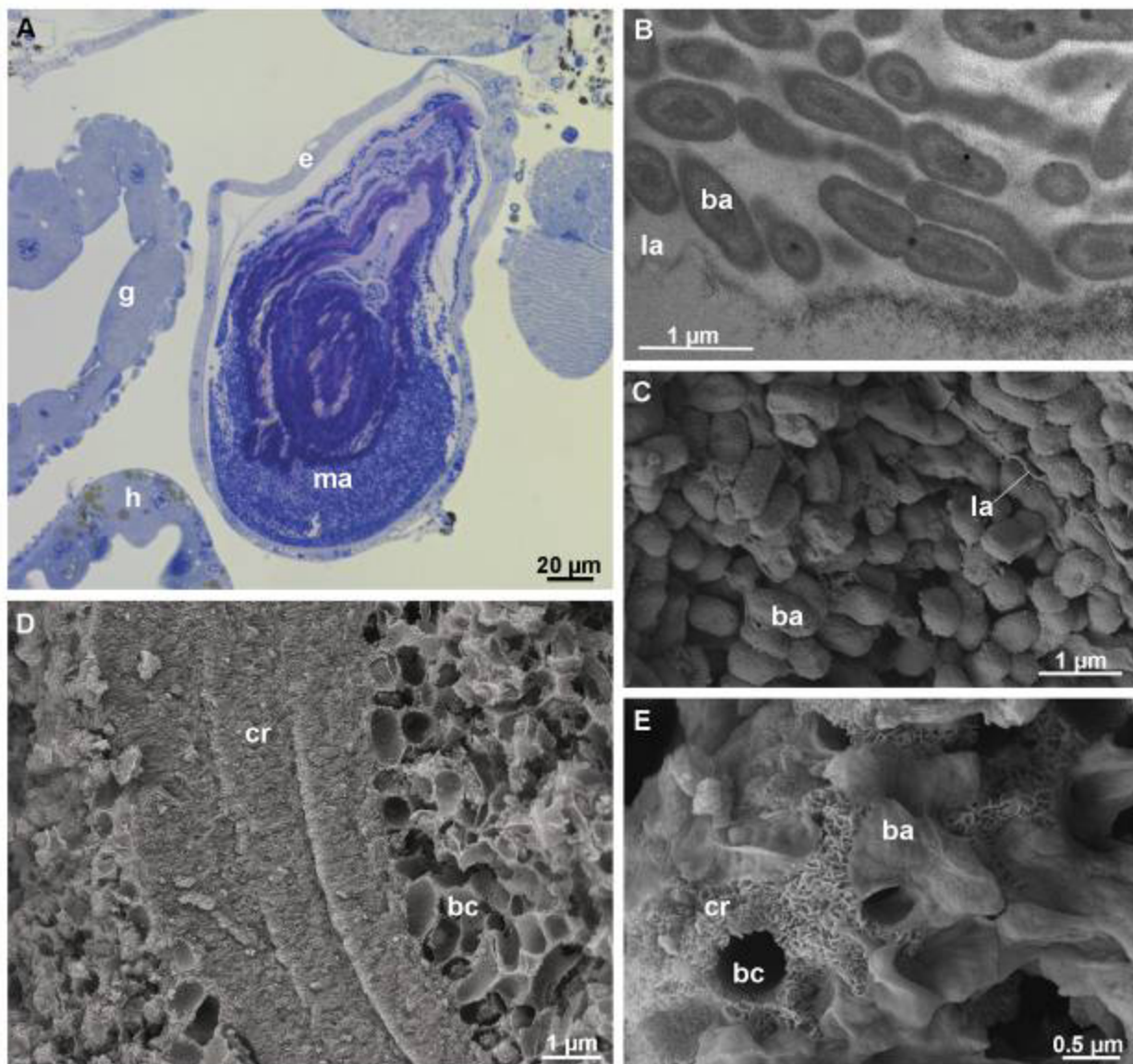


Figure 2. Structure and contents of the posterior calcium bodies in intermolt stage. (A) Semithin section through a posterior calcium body. A single-layered epithelium (e) encloses the layered, bacteria-containing matrix (ma). (B) Transmission electron micrograph showing bacteria (ba) in the proximity of a lamina (la) within a posterior calcium body. (C) Scanning electron micrograph (aldehyde/OsO₄ fixation) of the bacteria within the posterior calcium body. An extracellular lamina (la) is visible among the bacteria. (D) Scanning electron micrograph of the matrix within the posterior calcium bodies (methanol fixation). Layers of crystals (cr) and numerous bacterial casts (bc) are visible. (E) Scanning electron micrograph of crystals (cr) within the posterior calcium bodies. g - gut, h - hepatopancreas.
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matrix that encloses a central cavity (Figs. 4 C, D). This cavity contains numerous plied laminae, the surfaces of which are covered with small spherules less than 1 μm in size (Fig. 4 E). A lamina also lines the inner surface of the continuous mineralized matrix. As visible with TEM, the laminae are 10 nm thick and electron dense particles are present among them. The spherules visible with SEM are not preserved during specimen preparation for TEM.

The anterior calcium body epithelium in the intermolt stage is ultrastructurally similar to the posterior calcium body epithelium in most respects. Numerous mitochondria and electron dense particles are present in the anterior calcium body epithelium (Fig. 4 G), and its apical side is lined by an envelope very similar to the

envelope in the posterior calcium bodies (Fig. 4 H). A notable difference between the two pairs of organs is that the apical plasma membrane of the anterior calcium body epithelium is not folded in the intermolt stage (Figs. 4 G, H).

3. Structural Dynamics of Calcium Bodies during the Molt Cycle

Premolt stage. In early premolt stage, as the epidermis is synthesizing the new epicuticle, a new envelope is secreted in both pairs of calcium bodies (Figs. 5 A, B). The new envelope is formed over dense plaques on short protrusions of the plasma membrane

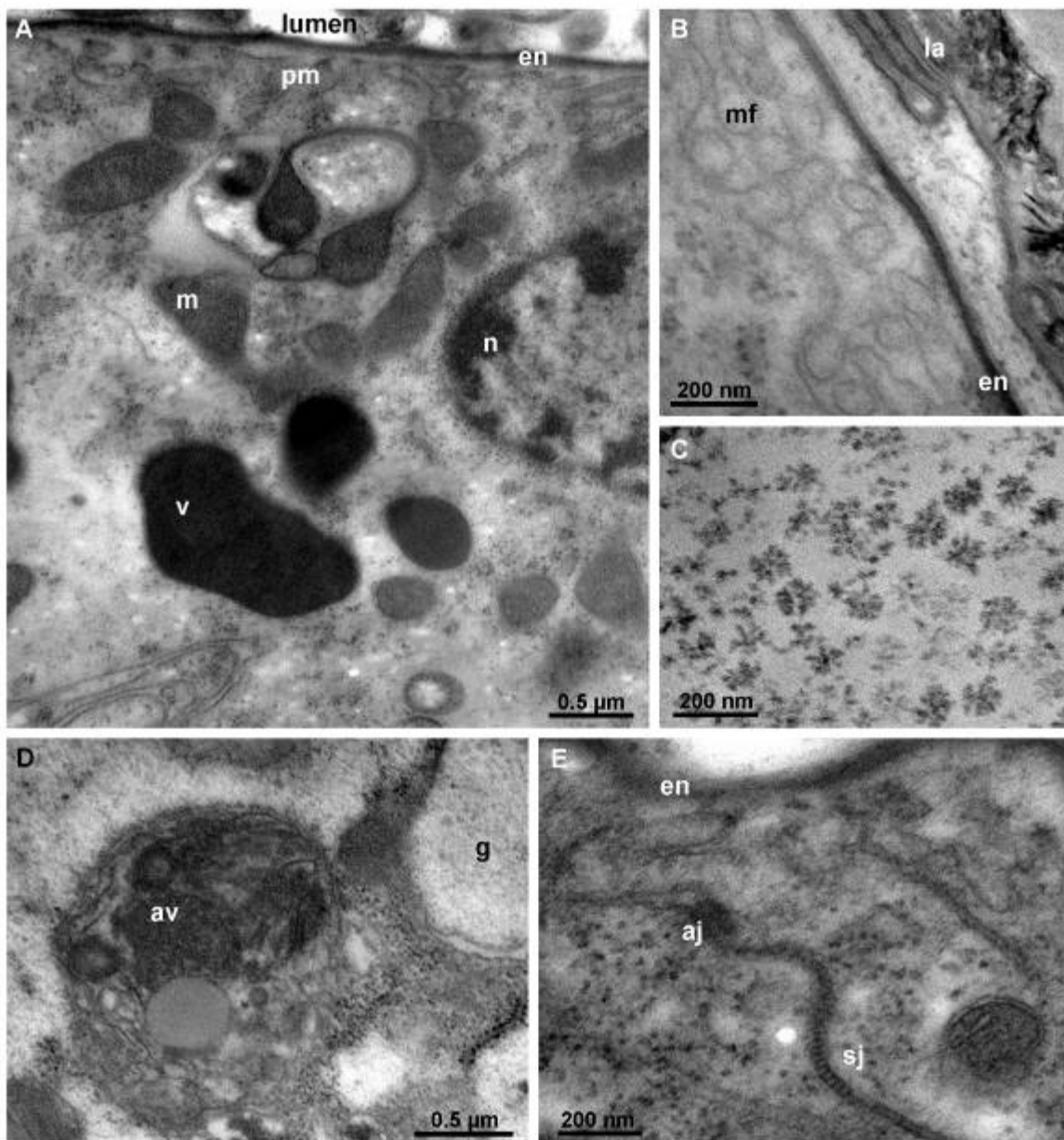


Figure 3. Ultrastructure of the posterior calcium body epithelium in intermolt stage. (A) Section through the epithelium (apical side up). An extracellular envelope (en) lines the folded apical plasma membrane (pm). Mitochondria (m) and electron dense vesicles (v) are present in the cytoplasm. (B) The apical surface of the posterior calcium body epithelium. The apical plasma membrane forms folds (mf). The envelope (en) consists of a proximal electron dense and a distal electron lucent layer. A lamina (la) is visible inside the lumen. (C) Rosettes of electron dense particles (most likely glycogen) in the cytoplasm of the epithelial cells. (D) An autophagic vacuole (av) in the cytoplasm of an epithelial cell. (E) Junction between two epithelial cells. The subapical adherens junction (aj) and the more basally positioned septate junction (sj) are visible. g – glycogen, n – nucleus.
doi:10.1371/journal.pone.0058968.g003

and is initially discontinuous (Fig. 5 A). In this respect, the process of deposition of the new envelope within the calcium bodies is ultrastructurally similar to the deposition of the new integumental epicuticle, which is secreted at the same time (Fig. 5 C). During the premolt stage, the apical plasma membrane is folded in the anterior calcium bodies as well (Fig. 5 D). In other respects, the ultrastructure of the premolt stage calcium body epithelia is similar to that of epithelia in the intermolt stage.

After the new envelope is synthesized, additional mineralized matrix is formed in the space between the detached old envelope and the new envelope in both pairs of calcium bodies. In the posterior calcium bodies, a glassy layer of mineralized matrix develops in the space between the old envelope and the new one (Figs. 5 E, F). Small spherules may also form along the laminae positioned central to the old envelope (Fig. 5 F). In the anterior calcium bodies, the old envelope appears to be incorporated into

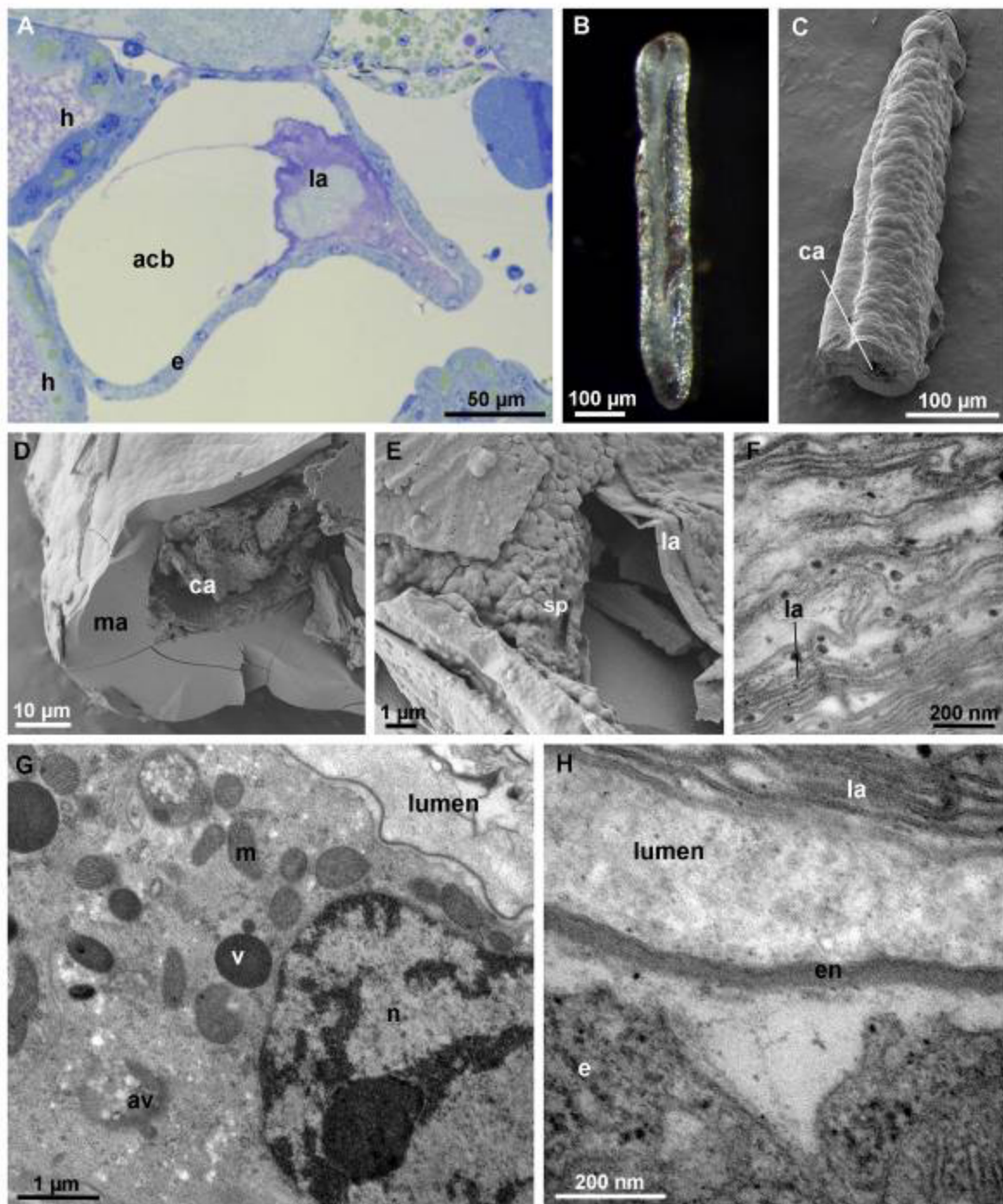


Figure 4. Anterior calcium bodies in intermolt stage. (A) Semithin section through an anterior calcium body (acb). An epithelium (e) forms the wall of the organ. An agglomeration of laminae (la) is visible in the lumen. Mineral content was lost during tissue preparation. (B) Mineral concretion from the medial tube of an anterior calcium body. (C) Scanning electron micrograph of a fractured mineral concretion from an anterior calcium body (methanol fixation) with a central cavity (ca). (D) Scanning electron micrograph of a fractured anterior calcium body (methanol fixation) showing a continuous layer of mineralized matrix (ma) formed around the central cavity (ca) with folded laminae. (E) Higher magnification scanning electron micrograph showing small spherules (sp) on the surface of laminae (la) within the central cavity. (F) Transmission electron micrograph of the laminae (la) within an anterior calcium body. (G) Cross-section through the anterior calcium body epithelium. The cytoplasm of epithelial cells contains numerous mitochondria (m), electron dense vesicles (v) and autophagic vacuoles (av). The apical plasma membrane is not folded. (H) The apical surface of an epithelial cell (e). The apical plasma membrane is lined by an envelope (en) consisting of a proximal electron dense and a distal electron lucent layer. Numerous laminae (la) are visible in the lumen. h – hepatopancreas, n- nucleus.
 doi:10.1371/journal.pone.0058968.g004

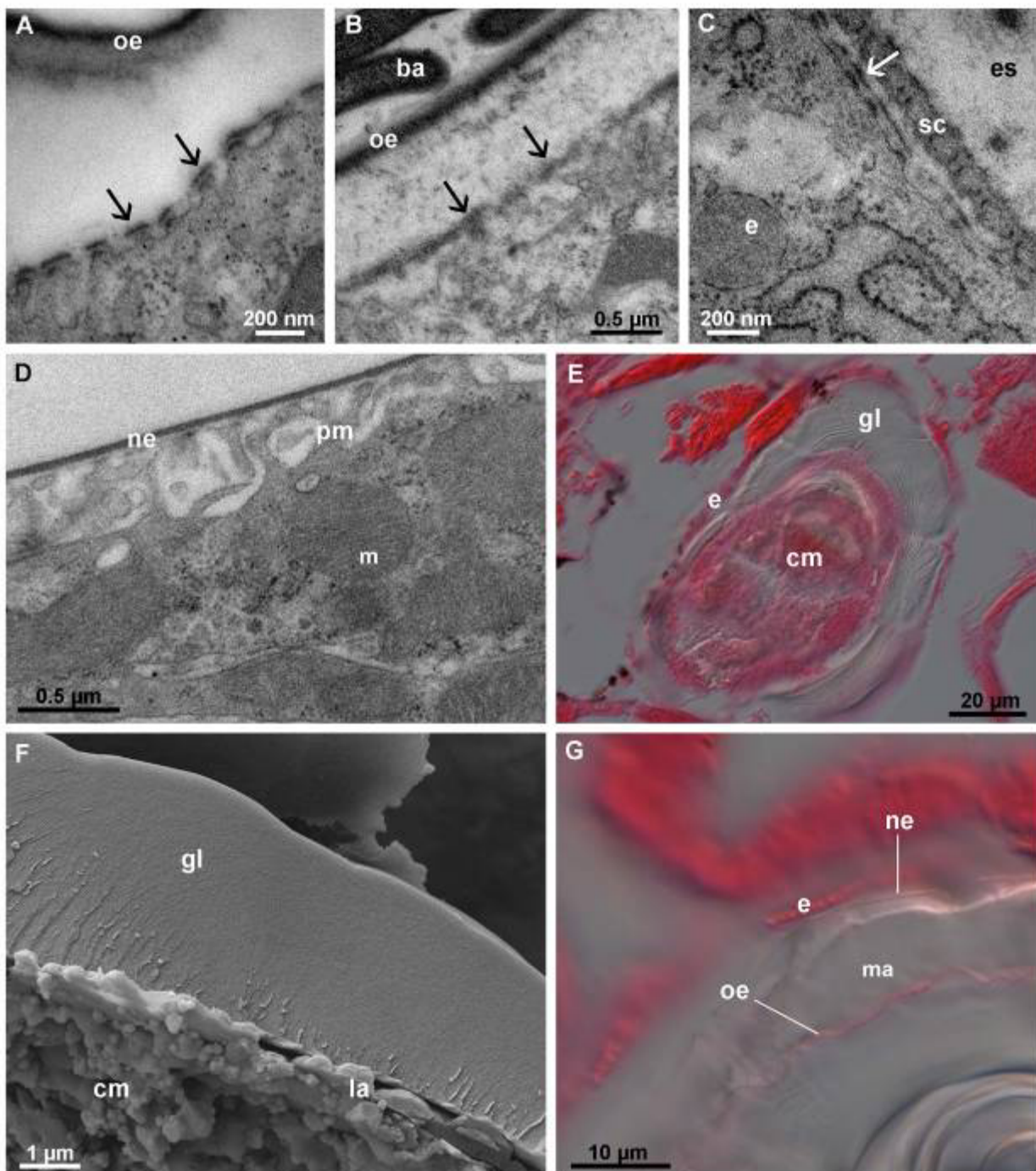


Figure 5. Calcium bodies in premolt stage. (A) Synthesis of the new envelope (arrows) in an anterior calcium body. The new envelope is discontinuous and is deposited over short protrusions of the apical plasma membrane. The old envelope (oe) is detached. (B) Synthesis of the new envelope (arrows) in the posterior calcium bodies. (C) Synthesis of the tergal epicuticle (arrow) in early premolt stage. A forming cuticular scale (sc) is visible. (D) The apical surface of the anterior calcium body epithelium in late premolt stage. The folded apical plasma membrane (pm) is lined by the new envelope (ne). (E) Differential interference contrast image of a posterior calcium body in late premolt stage (eosin stain). A glassy layer (gl) of mineralized matrix is present between the bacteria-containing central matrix (cm) and the epithelium (e). (F) Scanning electron micrograph of the glassy layer (gl) in a fractured posterior calcium body (methanol fixation). Small spherules are visible on the laminae (la) in the outer parts of the central matrix. (G) Differential interference contrast image of a premolt stage anterior calcium body (eosin stain). The old envelope (oe) is embedded in the mineralized matrix (ma). The new envelope (ne) lines the epithelium (e). ba – bacterium, es – ecdysial space, h – hepatopancreas, m – mitochondrion.
 doi:10.1371/journal.pone.0058968.g005

the forming mineralized matrix (Fig. 5 G), which resembles the matrix present within the lumen in intermolt stage.

Intramolt stage. The ultrastructure of the posterior calcium body epithelium in intramolt stage specimens appears unaltered with respect to the premolt stage epithelium (Fig. 6 A). By contrast, the apical epithelial surface in the anterior calcium bodies in intramolt stage is greatly enlarged by the formation of numerous membrane folds (Fig. 6 B). In addition, electron dense granules 50–100 nm in diameter are present in the intercellular spaces between adjacent epithelial cells (Fig. 6 B). These granules are formed on short protrusions of the lateral plasma membrane (Fig. 6 C) and are present basally to the subapical cell junctions.

The material within calcium bodies in intramolt stage is similar to that found in late premolt stage, i.e. the matrix of the anterior calcium bodies is extensive (Fig. 6 D) and the glassy layer in the posterior calcium bodies is well developed (not shown). However, the outer surface of the mineralized matrix formed during the

pre-molt stage in anterior calcium bodies appears etched, which suggests that the matrix is being dissolved in intramolt stage. Radially oriented columns of small granules (about 30 nm in diameter) are visible on the etched surface (Figs. 6 D, E).

Postmolt stage. During the first day following anterior ecdysis, the epithelial cells in both pairs of calcium bodies have a deeply folded apical membrane, electron dense granules in the basolateral parts of the spaces between adjacent cells are numerous (Figs. 7 A–E). At this time, the glassy layer is still present within the posterior calcium bodies and the anterior calcium bodies contain variable amounts of mineralized matrix (Figs. 7 F, G).

Two days after the anterior ecdysis, the ultrastructure of the calcium body epithelium is restored to its intermolt stage condition (Figs. 8 A, B). Electron dense granules are absent from the intercellular spaces between epithelial cells and adjacent plasma membranes are in close apposition. In anterior calcium bodies, the apical plasma membrane is smooth and lacks invaginations,

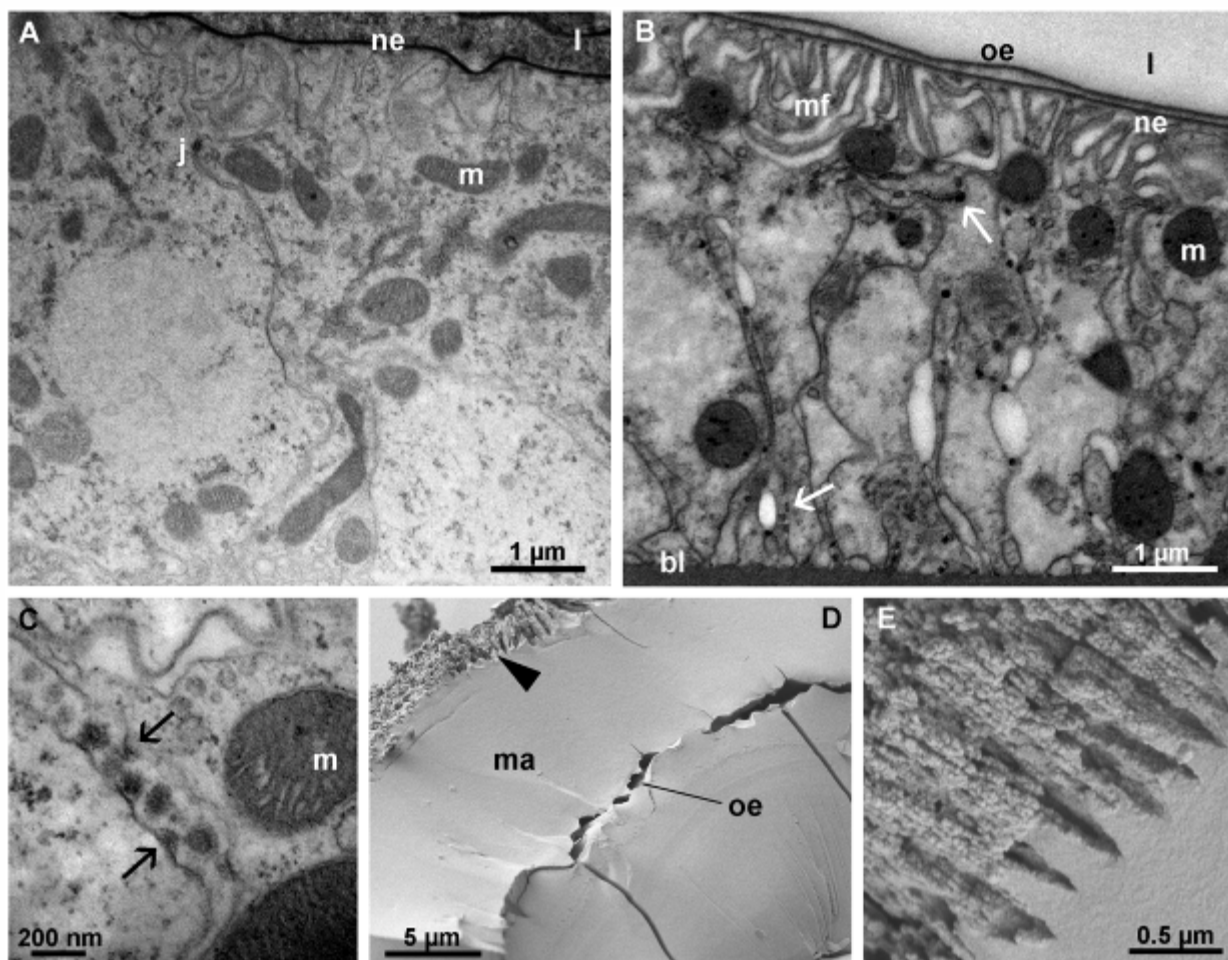


Figure 6. Calcium bodies in intramolt stage. (A) The posterior calcium body epithelium is unaltered with respect to the intermolt stage. (B) The anterior calcium body epithelium forms deep apical membrane folds (mf). Electron dense granules (arrows) are present in intercellular spaces between neighboring cells. Mineral components were lost during tissue preparation. (C) Formation of electron dense granules in intercellular spaces of the anterior calcium body epithelium on short protrusions (arrows) of the plasma membrane. (D) The mineralized matrix (ma) in an anterior calcium body. The outer surface of the mineral concretion (upper left side of image) appears etched (arrowhead). The embedded old envelope (oe) separates two layers of the mineralized matrix (ma). (E) Higher magnification of the etched surface of the mineralized matrix within an anterior calcium body showing granular material arranged in radial threads. The surface of the concretion is to the upper left side of the image. bl – basal lamina, j – cell junction, l – calcium body lumen, m – mitochondrion, ne – new envelope, oe – old envelope.
doi:10.1371/journal.pone.0058968.g006

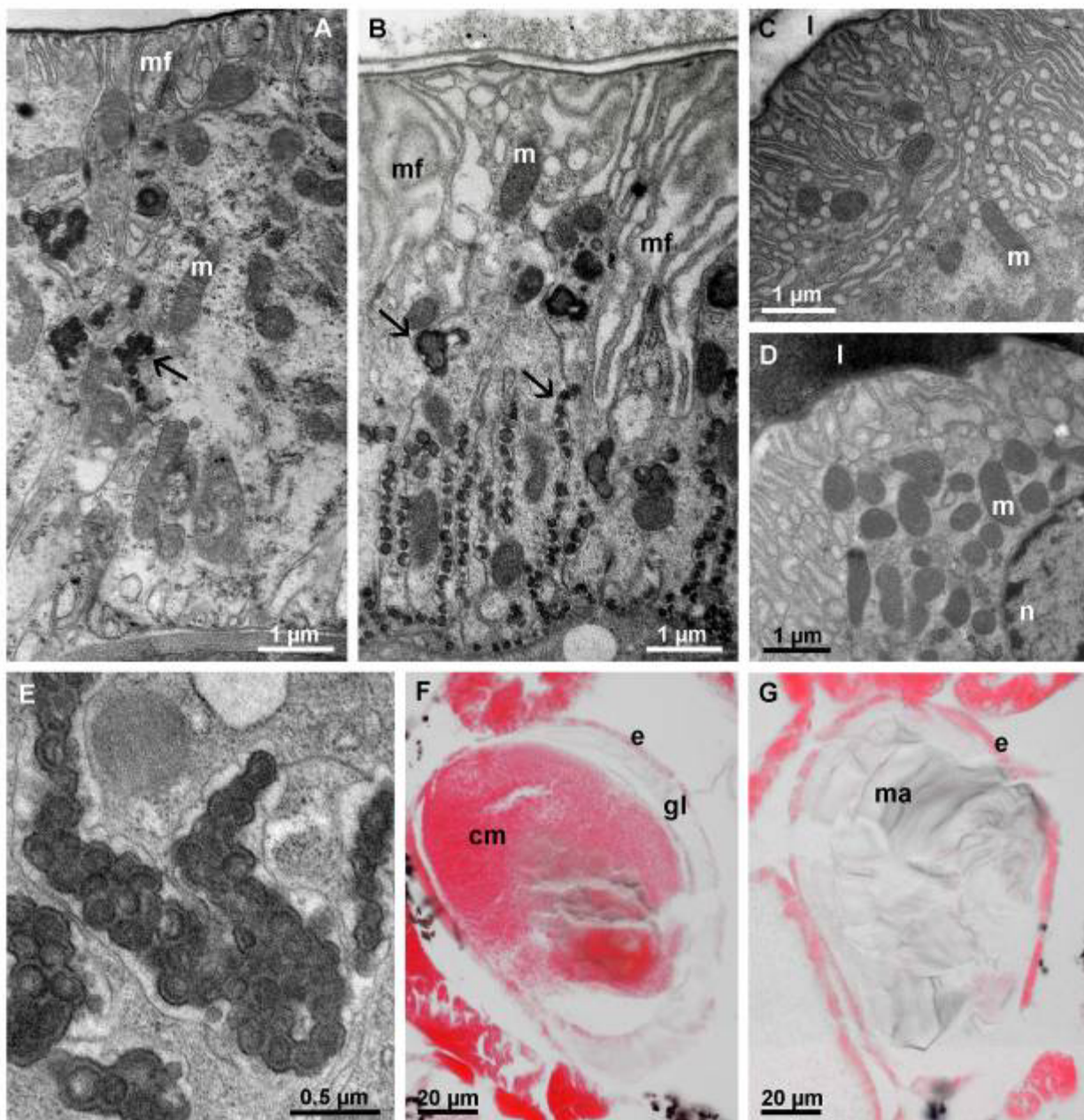


Figure 7. Calcium bodies one day after anterior ecdysis. (A) The posterior calcium body epithelium (apical side up) with apical membrane folds (mf) and electron dense granules (arrow) in intercellular spaces between neighboring cells. (B) The anterior calcium body epithelium (apical side up). Folds (mf) of the apical plasma membrane reach deeply into the epithelium. Electron dense granules of varying sizes (arrows) are present in intercellular spaces. (C) Oblique section through the apical part of the anterior calcium body epithelium showing numerous folds of the apical plasma membrane. (D) Oblique section through the apical part of the posterior calcium body epithelium with numerous membrane folds and mitochondria (m). (E) High magnification image of the electron dense granules in intercellular spaces of the anterior calcium body epithelium. (F) Paraffin section of the posterior calcium body (eosin stain) showing the glassy layer (gl), which is still present after anterior ecdysis. (G) Paraffin section of the anterior calcium body (eosin stain) showing the presence of mineralized matrix (ma) within it. cm – central matrix, e – epithelium, l – lumen, n – nucleus. doi:10.1371/journal.pone.0058968.g007

whereas in the posterior calcium bodies, the apical membrane folds are still present. The posterior calcium bodies lack a glassy layer at this stage; however, the bacteria and crystals remain in the central matrix (Fig. 8 C). The anterior calcium bodies, on the other hand, are completely devoid of the mineralized matrix and only numerous laminae remain in the lumen (Fig. 8 D), showing

that the mineralized matrix in anterior calcium bodies is completely degraded during the two days following exuviation.

4. Micro-Raman Spectroscopy

The Raman spectrum of the bacteria-containing central matrix within posterior calcium bodies (Fig. 9 A) demonstrates the

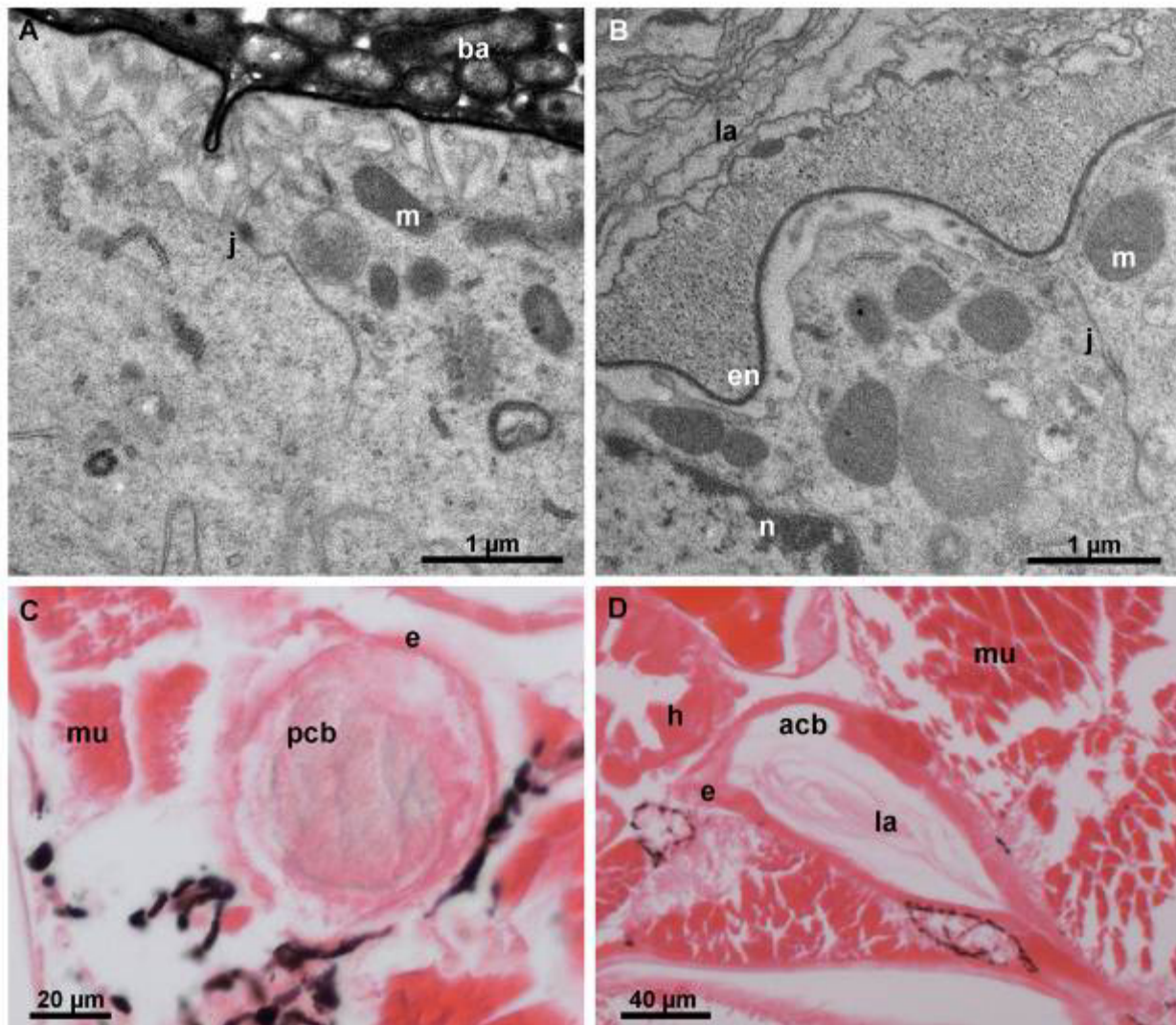


Figure 8. Calcium bodies two days after anterior ecdysis. (A) The posterior calcium body epithelium. Epithelial ultrastructure is similar to that of the intermolt stage epithelium. (B) The anterior calcium body epithelium. The apical surface of epithelial cells is no longer folded and adjacent cells are in close contact. (C) Paraffin section (eosin stain) of a posterior calcium body (pcb). No glassy layer is present. (D) Paraffin section (eosin stain) of an anterior calcium body (acb). The lumen is devoid of mineral and only organic laminae (la) are visible within it. ba – bacteria, e – epithelium, en – envelope, h – hepatopancreas, j – cell junction, m – mitochondrion, mu – muscle, n – nucleus.
doi:10.1371/journal.pone.0058968.g008

presence of carbonated apatite [14,15]. The prominent peak at 960 cm^{-1} corresponds to the ν_1 stretching of PO_4 [14,16] and the peak at 433 cm^{-1} to the ν_2 stretching of PO_4 in apatite [15]. The peak at 1077 cm^{-1} is contributed by the ν_1 stretching of CO_3 [14]. The demonstration of carbonated apatite is in accordance with the finding of crystals within the central matrix using SEM. At high frequencies the most prominent peak is located at 2935 cm^{-1} . This peak is associated with CH stretching and the peak at 1453 cm^{-1} with CH_2 wagging vibrations [17]. These peaks demonstrate the presence of large quantities of organic substances within the central matrix of posterior calcium bodies and can be attributed either to the abundant bacteria within the matrix [18] or to organic matrix components. The broad peak from 3000 to 3300 cm^{-1} corresponds to OH stretching of bound water [19].

The spectrum of the glassy layer, which develops in the posterior calcium bodies during premolt stage, is characterized by

three prominent peaks at 954 cm^{-1} , 987 cm^{-1} and 1080 cm^{-1} (Fig. 9 B). The peak at 954 cm^{-1} , attributed to ν_1 stretching of PO_4 , is characteristic of amorphous calcium phosphate (ACP). Unlike apatite, which features a narrow peak at about 960 cm^{-1} , the corresponding peak in ACP is broader and shifted towards smaller wavenumbers [15,16]. An additional peak of similar intensity at 987 cm^{-1} matches the ν_1 stretching of PO_4 in calcium hydrogen phosphates, i.e., brushite [20] and monetite [21]. However, the obtained spectrum does not allow us to draw firm conclusions about the presence of these minerals in the samples. If calcium hydrogen phosphates contribute to this peak, the presence of brushite is more likely, since its dehydration to monetite generally requires harsh conditions [22]. The peak at 1080 cm^{-1} is the strongest in the spectrum and, considering the broadness of this peak, is contributed by amorphous calcium carbonate [19]. The complexity of the spectrum in the $200\text{--}900\text{ cm}^{-1}$ region likely

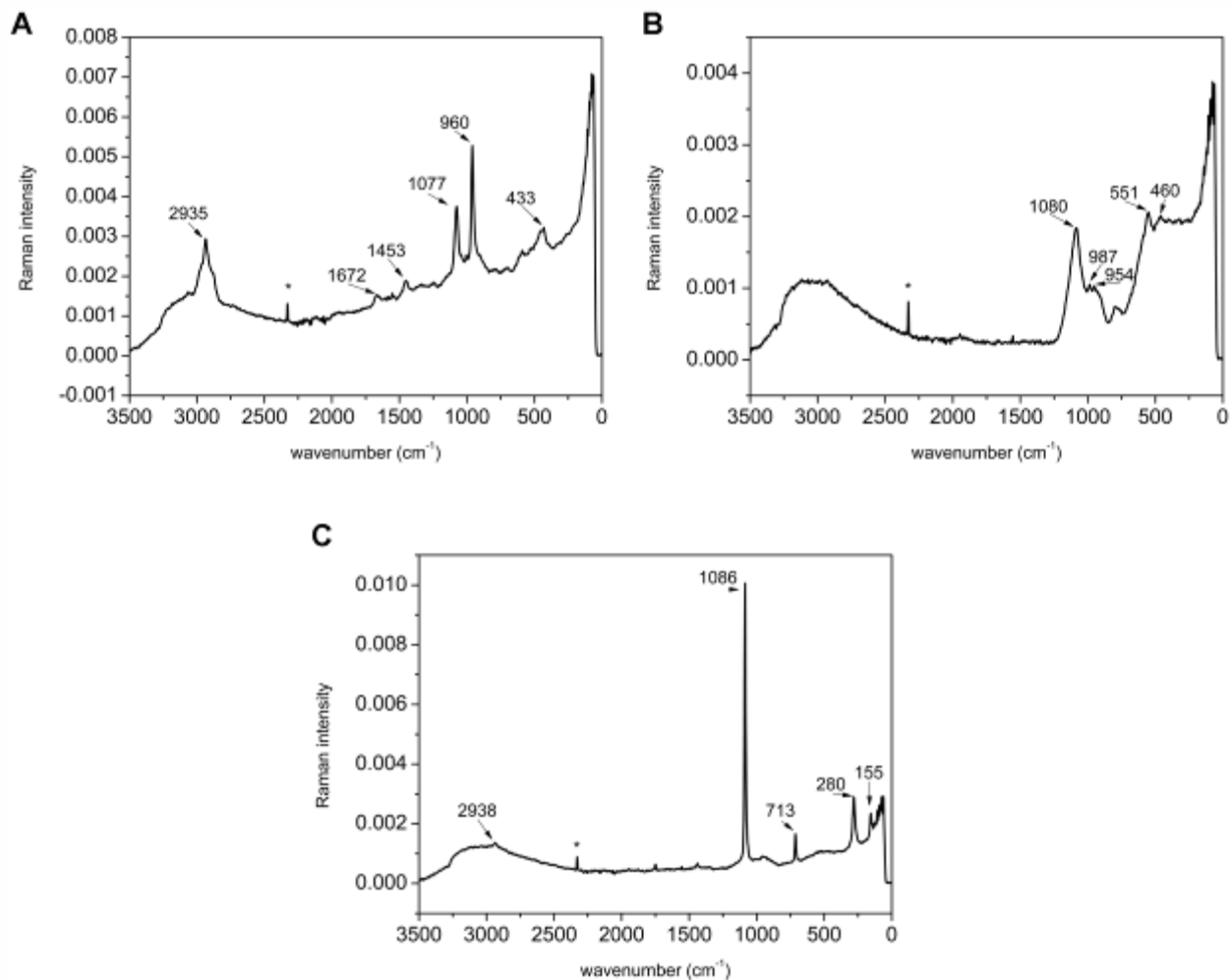


Figure 9. Raman spectra of mineralized matrices within calcium bodies. (A) Raman spectrum of the bacteria-containing central matrix in a posterior calcium body. (B) Raman spectrum of the glassy layer developed during the premolt stage in a posterior calcium body. (C) Raman spectrum of the concretion within an anterior calcium body. Positions of the most prominent peaks are given in cm^{-1} . Asterisks denote spikes.
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results from several mineral phases occurring together. Unlike the central matrix, the glassy layer of the posterior calcium bodies contains relatively small amounts of organic matrix components and consists mostly of minerals.

The Raman spectrum obtained from the mineralized matrix within the anterior calcium bodies (Fig. 9 C) demonstrates the presence of calcite, with peaks at 1086 cm^{-1} (symmetric ν_1 stretching of CO_3) [23], 713 cm^{-1} [19], 155 cm^{-1} and 280 cm^{-1} (calcite lattice vibration) [24]. The characteristic peaks at 155 cm^{-1} and 280 cm^{-1} distinguish calcite from amorphous calcium carbonate (ACC) [19] as well as vaterite and aragonite [25]. The spectrum lacks characteristic calcium phosphate peaks and peaks attributable to organic components are very small. Similar spectra were obtained from specimens in intermolt and intramolt stages. Analysis of the mineralized matrix in samples isolated in absolute ethanol and methanol gave identical results.

Discussion

The results of this study show that bacteria are present in calcium bodies of trichoniscids other than *T. albus*, in which they

were first reported [7], and are likely a general feature of calcium bodies. However, unlike *T. albus*, in which both pairs of calcium bodies harbor bacteria and are similar in structure and composition, only the posterior pair of calcium bodies contains bacteria in *H. riparius*, whereas the anterior pair is devoid of them.

There are two notable differences between the anterior and posterior calcium bodies of *H. riparius*. Firstly, the apical plasma membrane of the epithelium is deeply folded throughout the molt cycle in the posterior calcium bodies, whereas in the anterior pair it is smooth in intermolt stage and is deeply folded only during intramolt and postmolt stages and to a lesser extent during premolt stage, which may be linked to ion transport and matrix secretion. Secondly, the posterior calcium bodies continuously contain apatite and the transient glassy layer within them contains a high amount of calcium phosphate, whereas the mineralized matrix of the anterior calcium bodies contains only CaCO_3 -containing transient deposits. Our results are in agreement with the elemental analysis of calcium bodies in non-molting *H. riparius* [8], which demonstrated the presence of large amounts of phosphorus in the

posterior mineral concretions, whereas no phosphorus was detected in the anterior ones.

On the basis of our results we propose the following model of mineral deposition and resorption in the calcium bodies of *H. riparius*.

Before apolysis, deposition of a CaCO_3 -containing mineralized matrix begins around the laminae in the lumen of anterior calcium bodies. In the posterior calcium bodies, only the bacteria-containing matrix mineralized with apatite is present at this time. Later, the old envelopes detach from the calcium body epithelia and new envelopes are synthesized. These events are concomitant with integumental apolysis and the synthesis of the new epicuticle. Later in premolt stage, additional mineralized matrix is formed between the old envelope and the new envelope in both pairs of calcium bodies. In the anterior calcium bodies, the mineralized matrix contains only CaCO_3 , whereas in the posterior calcium bodies, the newly formed glassy layer contains a mixture of calcium carbonate and calcium phosphate, mostly in amorphous form. After the posterior ecdysis, degradation of the mineralized matrix within the calcium bodies begins. The apical surface of the anterior calcium body epithelium enlarges by forming numerous membrane folds and calcium transport across the anterior calcium body epithelium begins, as indicated by the intense folding of the apical epithelial surfaces and the presence of numerous electron dense granules in spaces between epithelial cells. Following the anterior ecdysis, degradation of mineralized matrix and intensive translocation of calcium take place in both pairs of calcium bodies. Within two days after the anterior ecdysis, mineral resorption from calcium bodies stops and the intermolt ultrastructure of calcium body epithelia is restored. At this point, the anterior calcium bodies are devoid of mineral content. In the posterior calcium bodies, only the bacteria-containing central matrix with apatite crystals remains and the glassy layer is completely degraded.

The continuously folded apical plasma membrane of the posterior calcium body epithelium indicates a constant high rate of transepithelial transport in the posterior calcium bodies. This is probably linked to the maintenance and functional regulation of the large bacterial population within the posterior calcium bodies. Epithelia with a highly folded or microvillous apical surface are also known from organs housing prokaryotic symbionts in other invertebrates, such as cephalopods [26] and insects [27].

The presence of calcium phosphate in bacteria-containing calcium bodies in *H. riparius* (this study) and *T. albus* [7] on one hand, and the absence of calcium phosphate in bacteria-free calcium bodies in *H. riparius* on the other, indicates that the function of bacteria is linked to calcium phosphate dynamics. However, bacteria might function in either its formation [28] or its dissolution [29,30] and further investigation will be necessary to clarify their role.

Raman spectroscopy demonstrated calcite in concretions from the anterior calcium bodies. This result is unusual, since transient calcium deposits of crustaceans generally contain ACC [2]. It has been suggested that the amorphous nature of transient calcium deposits is functionally important, since ACC is highly soluble and thus readily resorbed when the need for calcium arises [1,31]. Preparation methods used in this study should prevent the crystallization of ACC [31,32] and amorphous minerals were well preserved in sternal deposits of *T. albus* (Fig. S2) and in the glassy layer of posterior calcium bodies. In crustaceans, ACC is generally stabilized by phosphate ions, proteins, and small organic molecules [2,23,33]. It is noteworthy that sternal deposits contain some phosphate and large amounts of organic material (Fig. S2), whereas no phosphate and comparatively small amounts of organic components were detected in the concretions within

anterior calcium bodies of *H. riparius*. If the CaCO_3 in anterior calcium bodies is amorphous *in vivo*, which is not excluded, it is possible that the heating during Raman spectroscopic measurement caused the crystallization of the samples. Whatever the *in vivo* condition, CaCO_3 is readily resorbed from anterior calcium bodies after ecdysis.

The posterior calcium bodies in *H. riparius* are structurally and functionally similar to the calcium bodies of *T. albus* [7]. The continuously present crystalline mineral at the center of the posterior calcium bodies is apatite, as is the case in *T. albus*, whereas the transient glassy layer contains a mixture of calcium carbonate and calcium phosphate. Previously, energy dispersive X-ray spectrometry showed large amounts of calcium and phosphorus in the glassy layer in calcium bodies of *T. albus*; however, the Ca:P ratio was higher than would be expected for either apatite or ACP alone [7], which indicated that a mixture of minerals was present in this layer. In *H. riparius*, ACC and ACP form the glassy layer. If brushite is also present, as indicated by Raman spectroscopy, it should be readily resorbed from calcium bodies since its solubility [22] is comparable to that of ACC [34]. Unlike the glassy layer, apatite crystals in posterior calcium bodies are always present and we could not establish any changes to the bacteria-containing central matrix during the molt cycle. The same was observed in *T. albus* [7]. The function of apatite in calcium bodies thus remains unknown.

Calcium phosphate has more rarely been reported in crustacean calcium deposits than calcium carbonate. It represents a small fraction of the minerals in sternal deposits of *Porcellio scaber* [35] and was also detected in the calcium concretions within the posterior midgut caeca of *Orchestia cavimana* [23]. Although the function of calcium phosphates in the posterior calcium bodies is not known at this point, it is possible that they represent a reservoir for phosphorus in addition to calcium. It has been shown that the tergal cuticle of terrestrial isopods contains measurable amounts of calcium phosphate [36,37,38,39], predominantly in the proximal cuticular layers, where it supposedly stabilizes calcium carbonate in amorphous form [39]. Calcium phosphate in the glassy layer of the posterior calcium bodies could therefore provide a source of phosphate ions necessary for the mineralization of the exoskeleton.

If we assume that the etched appearance of the mineralized matrix within calcium bodies and the presence of electron dense granules in intercellular spaces are indicative of calcium resorption, there is evidently a delay between the onset of mineral resorption in the anterior and the posterior calcium bodies. This could be linked to the biphasic molt, but if we relate this sequence of events to the composition of the minerals that are resorbed in the two pairs of calcium bodies, we can conclude that only calcium carbonate is resorbed during intramolt stage, while both calcium carbonate and calcium phosphate are resorbed during postmolt stage, the resorption of calcium phosphate perhaps being linked to the phosphate-stabilized ACC mineralization of the endocuticle [39].

The ultrastructural features of the calcium body epithelium during mineral resorption are nearly identical to those reported for the anterior sternal epithelium during the resorption of sternal deposits in other oniscids [13]. The deeply folded apical plasma membrane, an extensive intercellular network between adjacent epithelial cells and electron dense granules forming within these spaces on membrane protrusions, observable in *H. riparius*, are also characteristics of the anterior sternal epithelium during resorption of the sternal CaCO_3 deposits of terrestrial isopods [13,40]. The electron dense extracellular granules were shown to contain Ca, N [40] and P [35] in the terrestrial isopod *Porcellio scaber*, which indicates that they function in transepithelial calcium transport in this species. Considering these ultrastructural similarities, it is likely

that similar cellular processes enable the transepithelial transport of calcium in calcium bodies and in sternal deposits of isopods. Similar features were reported in the epithelium of the posterior midgut caeca during resorption of mineral concretions in the amphipod *Orechestia cavimana* [41] and the epidermis during resorption of cuticular calcium in the crab, *Holthuisiana transversa* [42]. This indicates that different crustacean groups utilize a similar mechanism of transepithelial calcium transport. Interestingly, electron dense granules were not observed during mineral resorption from calcium bodies in *T. albus* [7], which might be the consequence of the generally slower metabolism in this subterranean species [43] and the consequently lower intensity of calcium transport.

H. riparius is the only known terrestrial isopod lacking sternal CaCO_3 deposits apart from the tyloid *Helleria brevicornis*, which was reported to store calcium in the hemolymph [8]. Sternal deposits are utilized by representatives of all major lineages of terrestrial isopods, including Tyllidae [8], Ligidae [44,45], at least three members of Synocheta [8,46], and all studied Crinocheta [45]. Despite many different views on oniscidean phylogeny [47], the absence of sternal deposits in *Hyloniscus* is likely the result of secondary reduction considering their presence in so many oniscidean lineages. It is possible that the anterior calcium bodies functionally replace sternal CaCO_3 deposits in this species.

Complete reliance on internal calcium deposits may be advantageous from the perspective of the timing of mineral deposition and resorption processes. CaCO_3 deposits in the ecdysial space can only start forming after the onset of apolysis. By contrast, mineral can already be deposited in calcium bodies before this point in the molting cycle, and our results demonstrate that this is the case in anterior calcium bodies of *H. riparius*. Whether the matrix formed at this point is mineralized with dietary calcium or with calcium resorbed from the exoskeleton remains to be established. Furthermore, sternal deposits are necessarily degraded during a relatively short period in intramolt stage if the animal is to avoid losing them with the anterior exuviae. The time available for the resorption of sternal deposits is thus limited to this narrow timeframe, which is impractical, as much of the endocuticle, in particular of the anterior body-half, is synthesized after the anterior ecdysis and calcium is still required for its mineralization in postmolt stage [4]. Calcium from an internal deposit, on the other hand, can be resorbed after ecdysis and we demonstrated that resorption of minerals from both pairs of calcium bodies in *H. riparius* is most intensive during the postmolt stage. Furthermore, the chemical environment within an internal epithelial compartment may be more efficiently regulated compared to the ecdysial space, making the utilization of calcium bodies energetically favorable.

We could not establish any luminal connections between the calcium bodies and the digestive system. However, they are

located close to digestive organs and the basal lamina of the calcium body epithelium is often in direct contact with that of the hepatopancreas. It is therefore possible that ions are transported almost directly between the calcium bodies and components of the digestive system despite the absence of a luminal connection.

Our results demonstrate that new envelopes are formed by the calcium body epithelium before each molt. Since calcium bodies are closed compartments, this should lead to accumulation of old envelopes within the calcium body lumen unless they are degraded. Since numerous laminae are observable within the lumen of both pairs of calcium bodies, and the structure and thickness of these laminae are identical to the electron lucent sublayer of the calcium body envelopes, it is likely that they are indeed accumulated remnants of old envelopes, secreted during previous molts. The mineralized matrix formed in the premolt stage is closely associated with the old envelopes. It is therefore possible that the old envelopes function in the initiation of mineral deposition.

Supporting Information

Figure S1 Absence of sternal deposits in *Hyloniscus riparius*. (A) Ventral view of anterior pereonites in late premolt stage. No sternal deposits are visible on the anterior sternites (st). (B) Ventral view of anterior pereonites in intramolt stage. No sternal deposits are visible. (C) The anterior sternal epithelium in intramolt stage. The epithelium (e) lacks pronounced apical membrane invaginations, epithelial cells are in close contact with each other and no electron dense granules are visible in intercellular spaces. ct – cephalothorax, es – ecdysial space, j – cell junction, nc – new cuticle. (TIIF)

Figure S2 Raman spectrum of sternal deposits in *Titanethes albus*. Positions of the most prominent peaks are given in cm^{-1} . Asterisk denotes spike. The peaks at 957 cm^{-1} , 1081 cm^{-1} , and 2935 cm^{-1} are contributed by calcium phosphate, calcium carbonate, and organic components, respectively. (TIIF)

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Author Contributions

Conceived and designed the experiments: JŠ MV. Performed the experiments: MV AR UN. Analyzed the data: MV AR JG JŠ. Wrote the paper: MV JŠ JG.

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3. RAZPRAVA IN SKLEPI

3.1 SINTEZA KUTIKULE PRI TRIHONISCIDIH IN NOVI PODATKI O PROCESU SINTEZE KUTIKULE ČLENONOŽCEV

Sintezo kutikule pri jamski mokrici smo podrobno preučili s presevno elektronsko mikroskopijo. Ultrastrukturne značilnosti epiderma so med levitvijo jamske mokrice enake kot pri površinskih mokricah (Price in Holdich, 1980b; Ziegler, 1997) in pri drugih rakih (Koulish in Klepal, 1981; Buchholz C. in Buchholz F., 1989; Compère, 1995). Ultrastruktura epitelija bolj ustreza novejšim opisom (Ziegler, 1997), s starejšimi poročili pa je v manjšem soglasju. Tako Price in Holdich (1980b) pri vzorcih v zgodnji predlevitveni fazi navajata vakuoliziranost epiteljskih celic, ki je na naših preparatih nismo opazili in bi bila lahko posledica njune priprave vzorcev (Ziegler, 1997).

Po apolizi je apikalna plazmalema oblikovana v kratke izrastke z elektronsko gostimi področji, na katerih se sintetizira organski matriks. Najprej celice izločijo epikutikulo, ki je na začetku na večih mestih prekinjena. Nekateri avtorji menijo, da se epikutikula sintetizira v odsekih (Koulish in Klepal, 1981; Ziegler, 1997), kakor je s presevno elektronsko mikroskopijo videti na prečnih prerezih. Naši rezultati kažejo, da sinteza epikutikule poteka drugače. Na mikrografijah poševno prerezanega integumenta je vidno, da se epikutikula sintetizira v zvezni plasti, ki je perforirana. Med sintezo epikutikule se oblikujejo tudi epikutikularne luske. Rezultati naše raziskave ne podpirajo predhodnih interpretacij tega procesa, čeprav so mikrografije, objavljene v literaturi, podobne našim. Drugi viri navajajo, da luske nastanejo kot izvihki epikutikule, v katere se proksimalno dodajajo pregrade, zaradi česar se izvihki daljšajo (Price in Holdich, 1980a; Powel in Halcrow, 1984). V nasprotju s takšnim modelom nastajanja lusk smo na podlagi naših rezultatov zaključili, da luske nastajajo na vzporednih izrastkih apikalne membrane, ki sta jih opazila že Powel in Halcrow (1984), in so jasno vidni na naših mikrografijah. Celični izrastki v nastajajoči luski omo gočajo dodatno nalaganje matriksa in debelitev luske, ki se zgodi pozneje med sintezo epikutikule. Tega procesa predhodne razlage tvorbe lusk ne predvidevajo, čeprav očitno poteka. Pregrade, ki so vidne v luskah na začetku njihove sinteze so najverjetneje zgolj deli matriksa epikutikule med sosednjimi celičnimi izrastki.

Pozneje v predlevitveni fazi se razmeroma hitro izoblikuje eksokutikula (eksokutikula jamske mokrice se sintetizira v dobrem tednu, medtem ko sinteza epikutikule pri tej vrsti traja okoli pet tednov). Med sintezo eksokutikule so v apikalni citoplazmi epiteljskih celic prisotni elektronsko gosti vezikli, ki so jih opazili tudi pri drugih vrstah rakov (Koulish in Klepal, 1981; Ziegler, 1997) in so verjetno sekrecijski. V tej fazi smo opazili tudi izrazitejšo nagubanje apikalne plazmaleme. Ko se eksokutikula debeli, postajajo daljši in izrazitejši tudi porni kanali ter celični izrastki v njih. Sinteza eksokutikule v sprednjem delu telesa nekoliko zaostaja za sintezo v zadnjem delu. Po levitvi posameznega dela telesa

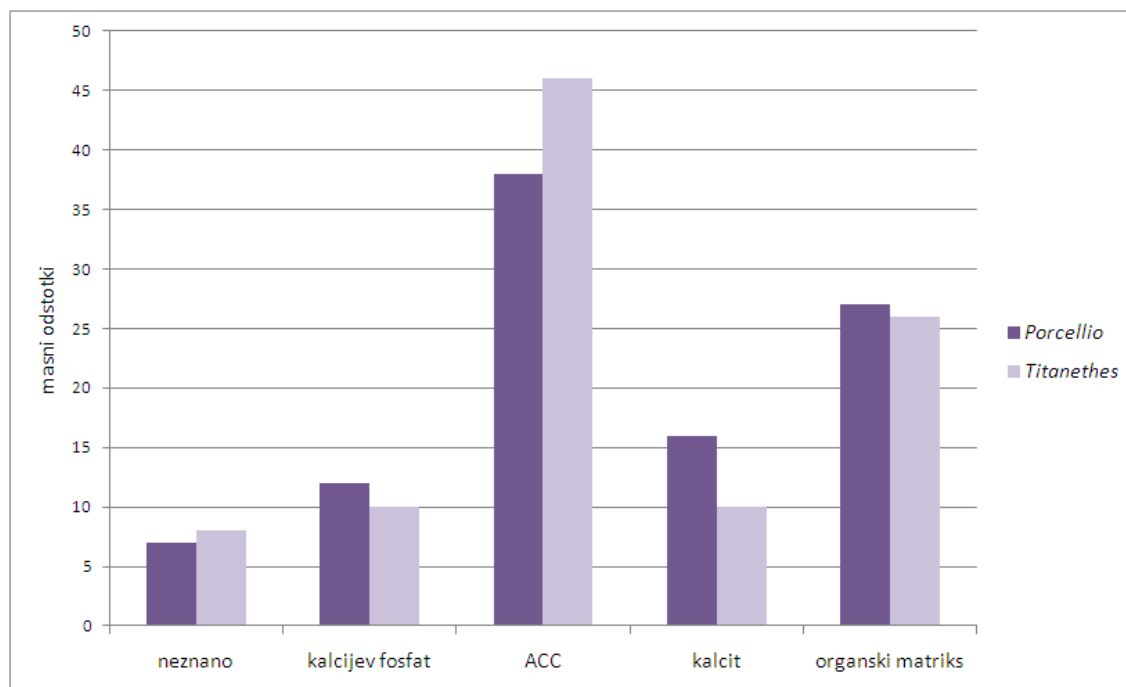
poteče sinteza endokutikule razmeroma hitro, zgolj v enem tednu. Endokutikula se prav tako sintetizira nad elektronsko gostimi področji na kratkih izrastkih apikalne plazmaleme. V polevitveni fazi postanejo porni kanali manj elektronsko gosti, kot so bili med sintezo eksokutikule in so na mikrografijah neizraziti.

Naši rezultati kažejo, da sta se med prilagajanjem jamske mokrice na podzemeljsko okolje zmanjšali zgolj pogostost levitev in hitrost sinteze kutikule, ki pri jamski mokrici poteka dva- do štirikrat počasneje kot pri površinskih mokricah (Steel, 1980), medtem ko so celični procesi, povezani s sintezo kutikule podobni kot pri drugih vrstah, pri katerih so bili opisani. Zanimivo je nagubanje apikalne plazmaleme epidermalnih celic jamske mokrice v pozni predlevitveni fazi, ki do sedaj pri enakonožcih ni bilo opisano, vendar je res, da se je večina dosedanjih raziskav (Ziegler, 1997; Glötzner in Ziegler, 2000; Štrus in Blejec, 2001) ukvarjala s sterniti in ne s tergiti. Podobno nagubanje je bilo opaženo med sintezo eksokutikule pri vrsti krila *Euphausia superba* (Buchholz C. in Buchholz F., 1989). Funkcija nagubanja apikalne plazmaleme ni jasna, lahko pa odraža pospešeno izmenjavo snovi z levitvenim prostorom, morda v povezavi z resorpcijo snovi iz stare kutikule.

Pri žuželkah prihaja po levitvi do dodatnih strukturnih sprememb kutikule (Wigglesworth, 1948). Tudi pri kopenskih enakonožcih je Compère (1990) opisal nastanek dodatnih plasti epikutikule z vrivanjem novih plasti med že obstoječe v polevitveni fazi. Podobnih sprememb epikutikule po levitvi pri jamski mokrici nismo opazili, kar je lahko povezano z razmeroma preprosto zgradbo epikutikule jamske mokrice. Epikutikulo sestavljata notranja in zunanja epikutikula, slednja pa pri jamski mokrici ni slojevita (Hild in sod., 2009), medtem ko pri drugih kopenskih enakonožcih zunanjo epikutikulo običajno sestavlja več slojev. Pri *O. asellus* je bilo npr. opisanih sedem slojev zunanje epikutikule (Ansenne in sod., 1988; Compère, 1990). Večslojna naj bi bila tudi zunanja eksokutikula pri rodu *Armadillidium* (Ansenne, 1988). Pri *H. riparius* smo opazili dodaten sloj v zunanji epikutikuli (Priloga A) in vse kaže, da se tudi pri tej vrsti dodatni sloj oblikuje po levitvi (osebno opažanje). Odsotnost modifikacij epikutikule po levitvi je torej med preučeni mi značilna zgolj za jamsko mokrico. Do strukturnih sprememb epikutikule po levitvi prav tako ne pride pri obrežni rakovici *Carcinus maenas* (Compère, 1995) ter krilu *Euphausia superba* (Buchholz C. in Buchholz F., 1989). Morda sta odsotnost modifikacij epikutikule po levitvi in njena preprosta zgradba posledica amfibijskega načina življenja jamske mokrice.

Price in Holdich (1980b) omenjata deformacijo eksokutikule po levitvi, ki naj bi jo povzročila sinteza endokutikule. Tudi tega pojava nismo opazili pri jamski mokrici, pri kateri je nova eksokutikula pred levitvijo in po njej videti enako. Morda sprememba strukture eksokutikule pri navadnem pasavčku (*Armadillidium vulgare*), ki sta ga preučevala Price in Holdich, izhaja iz mineralizacije tega sloja s kalcitom (Hild in sod., 2008). Kalcitni sloj namreč pri pasavčku sega mnogo globlje v eksokutikulo kot pri jamski

mokrici (Hild in sod., 2009).

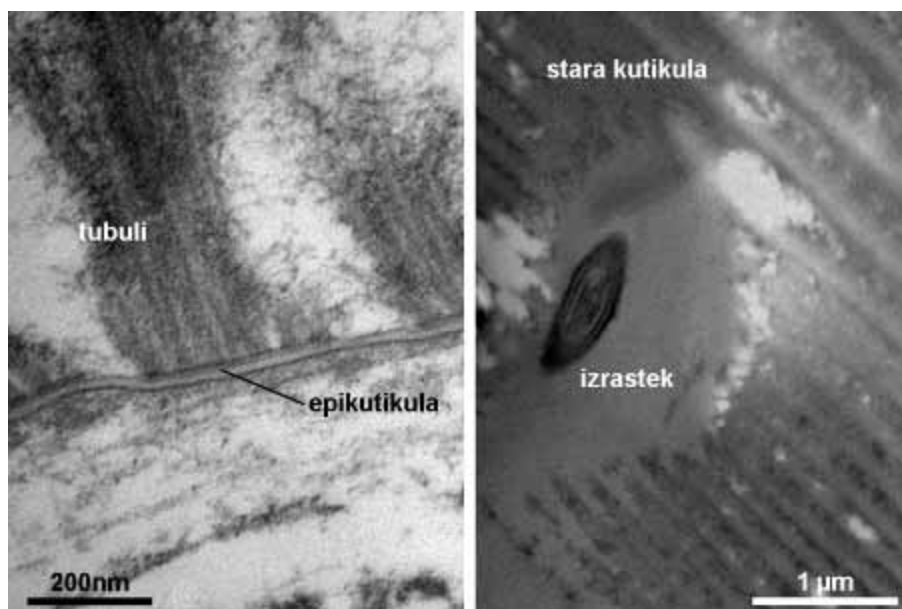


Slika 6: Primerjava deležev posameznih kemijskih komponent v kutikuli jamske mokrice (*Titanethes albus*) in navadnega prašička (*Porcellio scaber*).

Figure 6: Comparison of relative amounts of different chemical components in the cuticles of *Titanethes albus* and *Porcellio scaber*.

Kot kaže, med površinskimi in jamskimi mokricami ni velikih razlik v zgradbi kutikule integumenta. Primer jamske mokrice se ne sklada z ugotovitvami, da je kutikula integumenta jamskih členonožcev tanjša in manj mineralizirana kot pri površinskih živalih. Kutikula integumenta jamske mokrice (Hild in sod., 2009) je mineralizirana enako močno kot pri navadnem prašičku (Neues in sod., 2007), t.j., enak delež celokupne suhe mase kutikule predstavljajo mineralne komponente. Sestavi kutikule jamske mokrice in navadnega prašička sta skoraj enaki (slika 6), večje razlike pa se pojavljajo med različnimi površinskimi vrstami (Neues in sod., 2007). Hild in sod. (2009) so zaključili, da vsebuje kutikula jamske mokrice manj magnezija, kot ga najdemo pri drugih enakonožcih. Kutikula jamske mokrice (~9 μm pri 12 mm dolgi živali) je tanjša kot pri primerljivo velikih predstavnikih skupine Crinocheta (Hild in sod., 2008), vendar je možno, da imajo predstavniki skupine Crinocheta debelejšo kutikulo zaradi svoje evolucijske zgodovine ali naseljevanja bolj sušnih habitatov od trihoniscidov (Schmidt, 2008). Podatki, ki so na razpolago, ne dopuščajo dokončnih zaključkov glede tega, ali je kutikula jamske mokrice tanjša od preučenih predstavnikov skupine Crinocheta zaradi prilagoditve na jamsko okolje, zaradi amfibijskega načina življenja ali zaradi posebnosti skupine Synocheta. Podatkov o kutikuli drugih skupin enakonožcev ni veliko, če pa debelino kutikule ocenimo

na podlagi slikovnega materiala v objavljenih publikacijah (Žnidaršič in sod., 2012), je kutikula pri pobrežni mokrici *Ligia italica* debela približno toliko kot pri jamski mokrici. Kutikula *H. riparius* je tanjša od kutikule jamske mokrice (slika 2), vendar je *H. riparius* tudi precej manjši (telesna dolžina do 5 mm).



Slika 7: Tubuli in celični izrastki v levitvenem prostoru jamske mokrice v medlevitveni fazi. Levo: pripenjanje tubulov na epikutikulo. Desno: celični izrastek, ki sega v staro kutikulo.

Figure 7: Tubules and cellular extensions in the ecdysial space of *Titanethes albus* in intramolt stage. Left: the attachment of tubules to the epicuticle. Right: a cellular extension reaching into the old cuticle.

V levitvenem prostoru jamske mokrice smo v predlevitveni in medlevitveni fazi opazili šope tubulov, debelih okoli 20 nm. Zunajcelični tubuli nanometerske debeline so bili opaženi v levitvenem prostoru tudi pri nekaterih drugih mokricah. Na sternitih sta jih npr. opazila Glötzner in Ziegler (2000) pri vrsti *Ligidium hypnorum*, pri pobrežni mokrici *Ligia italica* pa o njih poročajo Štrus in sod. (2003). Pri pobrežni mokrici so opazili, da so tubuli lahko povezani z mineraliziranimi sferulami v levitvenem prostoru (Štrus in Tušek-Žnidarič, 2005). Tubuli so zunajcelične strukture, pritrjajo se na epikutikulo in jih verjetno sestavljajo proteini. Njihova funkcija ni jasna. Glötzner in Ziegler (2000) sta predlagala, da pomagajo pri vezavi vode, vendar te domneve nista podprla. Zelo podobne, a krajše (do 400 nm dolge) tubularne strukture prekrivajo dorzalno površino kutikule pri morskem ceponožcu *Porcellidium viride* (Gharagozlou-van Ginneken in Bouligand, 1975). Zanimivo je, da pri jamski mokrici tubuli niso razporejeni po celotni površini nove kutikule, ampak se običajno pojavljajo v šopih v povezavi s celičnimi izrastki, ki segajo v levitveni prostor. Naknadne raziskave so pokazale, da celični izrastki v sredini šopov tubulov segajo vse do stare kutikule (slika 7). Povsem možno je, da gre v resnici za dendrite še neznanega kutikularnega čutila. Če gre za dendrične izrastke, je v čutilu

prisoten zgolj en dendrit, obdaja pa ga, kot kaže, ena sama ovojna celica. V elektronsko gosto ovojnico ovit izrastek, ki sega skozi levitveni prostor, lahko predstavlja senzorično povezavo morebitnega čutila s staro kutikulo v predlevitveni fazi. Takšne povezave med starimi in nastajajočimi senzilami obstajajo med levitvijo pri deseteronožcih (Kouyama in Shimozawa, 1984), pri enakonožcih pa do sedaj še niso bile opažene. Price in Holdich (1980b) sta predpostavila, da se senzorične povezave s staro kutikulo v predlevitveni fazi pri kopenskih enakonožcih prekinejo. Naši rezultati tega ne potrjujejo, a tudi nedvoumno ne ovržejo. Smiselno bi bilo nadaljnje delo na tem problemu s serijskimi ultratankimi rezinami integumenta živali v predlevitveni fazi.

3.2 KALCIJEVA TELESCA

Kalcijeva telesca so prisotna pri obeh obravnavanih vrstah rakov. Za veliko jamsko mokrico je ta podatek nov, vendar pričakovan, ker o prisotnosti kalcijevih telesc poročajo pri mnogih trihoniscidih (Verhoeff, 1926; Méhely, 1932), tudi pri nekaterih drugih jamskih predstavnikih te družine (Verhoeff, 1927). Kaže, da so kalcijeva telesca splošna značilnost trihoniscidov. Do sedaj (vključno s to raziskavo) so bila opažena pri 19 vrstah iz 9 rodov, med njimi pa so predstavniki obeh poddružin trihoniscidov, Trichoniscinae in Haplophthalminae (Tabela 1). Za nobenega trihoniscida do sedaj ni bilo ugotovljeno, da nima kalcijevih telesc.

Kalcijeva telesca so zaprte epiteljske vrečke, oblikovane v longitudinalno usmerjeno osrednjo cev z manjšim lateralnim izrastkom. Epitelij kalcijevega telesca na luminalni (apikalni) strani prekriva zunajcelična ovojnica, ki je tanjša od 500 nm. Lumen kalcijevega telesca zapolnjujejo številne bakterije, ki so razporejene v plasti, ločene z nekaj nanometrov debelimi laminami. Med bakterijami se pojavljajo kristali apatita, ki so bolj koncentrirani v osrednjem delu lumna in v bližini lamin.

Običajno so kalcijeva telesca prisotna v zadnjih treh členih pereona, segajo pa tudi v začetek pleona (Méhely, 1932; Ziegler, 2003). Med preučeniimi vrstami je izjema rod *Hyloniscus*, pri katerem anteriorni par kalcijevih telesc sega tudi anteriorno od 5. pereonita. Nahajajo se ventrolateralno v osrednji telesni votlini ob hepatopankreasu. Če naše rezultate primerjamo s predhodnimi poročili (Verhoeff, 1926; Méhely, 1932; Ziegler, 2003) in upoštevamo naše ugotovitve glede števila teh organov pri *H. riparius*, za katerega si podatki iz literature nasprotujejo (Méhely, 1932; Ziegler, 2003), lahko zaključimo, da imajo trihoniscidi po dva para kalcijevih telesc.

Tabela 1: Prisotnost kalcijevih telesc pri trihoniscidih.

Table 1: Presence of calcium bodies in trichoniscids.

Takson	Vir
<i>Aegonethes cervinus</i>	Verhoeff, 1931
<i>Alpioniscus strasseri</i>	Verhoeff, 1927
<i>Androniscus</i>	
<i>dentiger</i>	Méhely, 1932
<i>roseus</i>	Ziegler, 2003
<i>Cyphoniscellus gotscheensis</i>	Méhely, 1932
<i>Haplophthalmus montivagus</i>	Ziegler, 2003
<i>Hyloniscus</i>	
<i>mariae</i>	Méhely, 1932
<i>riparius</i>	Méhely, 1932; Dudich, 1932; Ziegler 2003
<i>siculus</i>	Méhely, 1932
<i>transylvanicus</i>	Méhely, 1932
<i>vividus</i>	Méhely, 1932
<i>Tachysoniscus austriacus</i>	Méhely, 1932
<i>Titanethes albus</i>	Vittoni in sod., 2012
<i>Trichoniscus</i>	
<i>corniculatus</i>	Verhoeff, 1926
<i>noricus</i>	Méhely, 1932
<i>pygmaeus</i>	Méhely, 1932
<i>pussilus</i>	Ziegler, 2003

Rezultati nekaterih starejših raziskav, zlasti Méhelyjevega dela (1932), se glede števila in velikosti kalcijevih telesc od naših ugotovitev precej razlikujejo. To lahko v celoti pojasnimo, če upoštevamo metode, uporabljene v različnih raziskavah. Méhely je kalcijeva telesa preučeval z barvanjem celih živali, ki jih je zbistril in diferenciral v kisljih raztopinah. Zaradi uporabe takšnega postopka lahko predvidevamo naslednje: (1) da je v tkivu ostal in se obarval le izrazito netopen apatit, bolj topne kalcijeve soli, kot so kalcijevi karbonati in amorfni kalcijev fosfat (ACP), pa so se v kislem mediju raztopile; (2) da

dejanskih mej organa, ki jih predstavlja epitelij, ni bilo mogoče opaziti, ker so mehka tkiva med pripravo vzorcev postala prosojna; (3) da so bili posledično organi videti manjši, ker se skupki apatita nahajajo v osrednjem delu organa. Lateralni izrastki so bili lahko videti kot ločeni organi, ker so skupki apatita v njih ne stikajo nujno s skupki v osrednji cevi organa (npr. pri *H. riparius*).

Iz podobnih razlogov so naši rezultati glede števila in položaja kalcijevih telesc pri *H. riparius* drugačni od Zieglerjevih (2003). V naši raziskavi smo se ob mineraliziranem matriksu osredotočili tudi na epitelij kalcijevih telesc in organske komponente njihove vsebine, torej na kalcijevo telesce kot organ. To je glavna razlika v primerjavi s predhodnimi raziskavami, ki so se osredotočale izključno na minerale znotraj kalcijevih telesc in posledično dale drugačne rezultate glede števila in položaja kalcijevih telesc.

Kot je bilo pokazano pri *H. riparius*, izloči epitelij kalcijevega telesca novo ovojnico v zgodnji predlevitveni fazi, sočasno z začetkom izločanja epikutikule integumenta. Stara ovojnica se pred tem loči od epitelija kalcijevega telesca in okoli nje se oblikuje plast mineraliziranega matriksa, ki po levitvi ni več prisotna. Čeprav pri jamski mokrici sinteze nove ovojnice nismo uspeli opaziti, je potek dogodkov pri tej vrsti verjetno podoben in tudi tu se steklast sloj matriksa oblikuje okoli lamine, ki je najverjetneje prejšnja ovojnica. Številne tanke lamine v notranjosti kalcijevih telesc so zelo verjetno ovojnice, ki so se ločile od epitelija v predhodnih levitvah in se nato delno razgradile.

S presevno in vrstično elektronsko mikroskopijo smo ugotovili prisotnost številnih prokariotskih organizmov v kalcijevih telescih jamske mokrice in v posteriornih kalcijevih telescih *H. riparius*. To ugotovitev smo dodatno podprli s hibridizacijo *in situ*, ki je potrdila prisotnost evbakterij v kalcijevih telescih. Kalcijeva telesca, ki vsebujejo bakterije, so z njimi popolnoma zapolnjena, bakterije pa so bile prisotne pri vseh pregledanih osebkih (okoli 30 osebkov jamske mokrice in okoli 60 osebkov *H. riparius*). Prisotnost bakterij v kalcijevih telescih je nepričakovan rezultat. Do sedaj pri rakih ni bil znan noben depozit kalcija, ki bi vseboval veliko število bakterij. Tako velika bakterijska populacija nedvomno močno vpliva na kemijsko okolje v kalcijevih telescih in njihova prisotnost ima skoraj gotovo funkcionalen pomen, vendar na podlagi podatkov, ki so trenutno na voljo, težko sklepamo o njihovi funkciji. Pri *H. riparius* smo ugotovili, da prisotnost bakterij sovpada s prisotnostjo kalcijevega fosfata v kalcijevih telescih. To nakazuje, da je funkcija bakterij povezana z dinamiko matriksa, mineraliziranega s kalcijevim fosfatom. Bakterije morda sodelujejo pri nalaganju kalcijevega fosfata in njegovi stabilizaciji v določeni obliki, pri čemer bi lahko imela pomembno vlogo bakterijska alkalna fosfataza, ki sprošča fosfatne ione iz organskih molekul (Hirschler in sod., 1990; Benzerara in sod., 2004). Ker bakterije kalcijeve ione, ki vdirajo v celico iz okolja, aktivno vračajo v medij ter s tem vzdržujejo nizko koncentracijo kalcija v celici, lahko pride do lokalnega koncentriranja kalcija na površini bakterijske celice, kjer se lahko

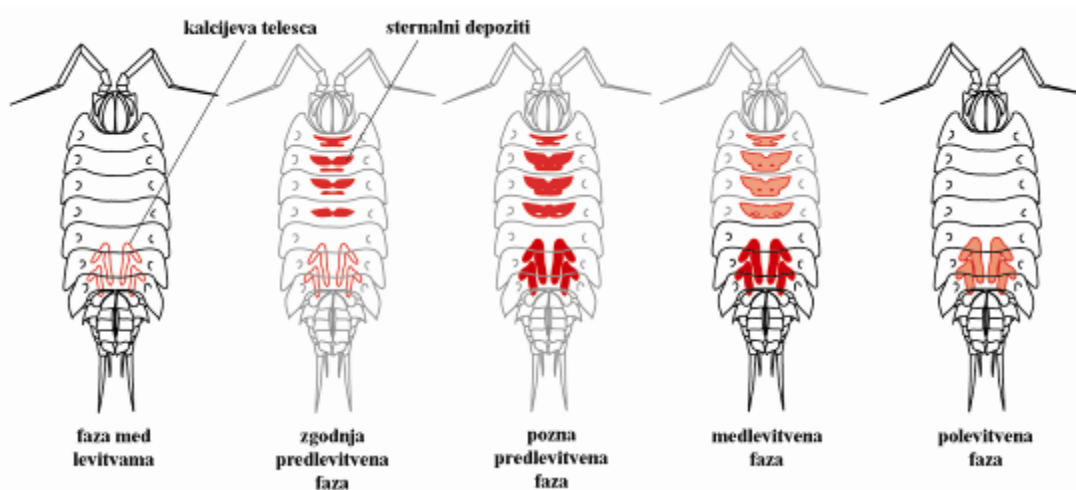
veže na kisle molekule na celični površini ali na fosfatne skupine membranskih fosfolipidov, s čimer se začne mineralizacija (Benzerara in sod., 2004; Rivadeneyra in sod., 2012). Rivadeneyra in sod. (2012) so pokazali nalaganje amorfnega kalcijevega fosfata v obliki sferul na površini bakterij rodu *Chromohalobacter*. Če je mineralizacija potekala dlje, se je okoli sferul ACP naložil tudi aragonit. Po drugi strani lahko bakterije sodelujejo tudi pri raztapljanju kalcijevega fosfata (Welch in sod., 2002), ki ga lahko povzročijo z zakisanim medijem (Welch in sod., 2002), verjetno pa tudi z aktivnim privzemanjem fosfatnih ionov in posledičnim nižanjem njihove koncentracije v raztopini (Smith in sod., 1978; Welch in sod., 2002) Bakterije bi lahko tudi prispevale organske komponente matriksa ali sodelovale pri njejovi razgradnji.

Odprto ostaja tudi vprašanje porekla epitelijev kalcijevih telesc in bakterij v njih med ontogenetskim razvojem. Jasno je, da so kalcijeva telesca pri odraslih osebkih zaprti epitelijski razdelki, vendar ne vemo, iz katere strukture se oblikujejo med ontogenetskim razvojem in tudi ne, na kakšen način ter kdaj se vanje naselijo bakterije. Pri drugih skupinah rakov se notranji depoziti kalcija navadno nahajajo v delih ali izrastkih prebavila, npr. v želodcu (gastroliti deseteronožcev; Travis, 1960) ali v izrastkih srednjega črevesa (*Orchestia*, *Niphargus*; Graf, 1961) in ne v zaprtih epitelijskih vrečkah. Prav tako ni jasno ali se bakterije prenašajo na potomce ali jih ličinke pridobijo iz okolja, ko zapustijo valilnik matere.

Za tiste enakonožce, pri katerih so prisotni zgolj sternalni depoziti, so postavili model skladiščenja kalcija in mineralizacije kutikule (Steel, 1993; Ziegler in sod., 2005). Model predvideva, da se v predlevitveni fazi kalcij resorbira iz kutikule zadnjega dela telesa in se skladišči v levitvenem prostoru sprednjega dela telesa pretežno v obliki ACC (Ziegler in sod., 2005). V medlevitveni fazi se depoziti v levitvenem prostoru sprednjega dela telesa resorbirajo, kalcij pa se po hemolimfi prenese v zadnji del telesa, kjer se porabi za mineralizacijo nove kutikule. Po anteriorni levitvi žival zaužije anteriorni lev (Ziegler in sod., 2005), v prebavilu iz njega resorbira kalcij in ga porabi za mineralizacijo sprednjega dela telesa. Pozneje se eksoskelet dodatno mineralizira s kalcijem, pridobljenim s hrano (Steel, 1993).

Živali s kalcijevimi telesci imajo na voljo dodaten depozit kalcija. Časovni potek dogodkov v levitvenem ciklu pri jamski mokrici shematično prikazuje slika 8. Sternalni depoziti so pri tej vrsti prisotni v enakem časovnem obdobju kot pri drugih mokricah (Messner, 1965; Steel, 1993), pojavijo se v zgodnji predlevitveni fazi, se dokončno razvijejo v pozni predlevitveni fazi in izginejo v medlevitveni fazi. V pozni predlevitveni fazi se razvije tudi dodaten steklast sloj v kalcijevih telescih, ki vsebuje tako kalcijev fosfat kot kalcijev karbonat. Na podlagi naših rezultatov smo oblikovali model premeščanja kalcija med levitvijo velike jamske mokrice.

V predlevitveni fazi se začne resorpcija kalcija iz stare kutikule in nalaganje kalcija v obliki sferul ACC v levitvenem prostoru sprednjega dela telesa. V pozni predlevitveni fazi, ko se v sprednjem delu telesa začne sinteza nove eksokutikule, se začne kalcij nalagati tudi v kalcijeva telesa, kjer med staro in novo ovojnico nastane steklasta plast mineraliziranega matriksa, ki vsebuje mešanico ACC in ACP. V medlevitveni fazi žival resorbira depozite v levitvenem prostoru in kalcij porabi za mineralizacijo kuikule. V polevitveni fazi žival resorbira steklasti sloj v kalcijevih telescih in z resorbiranim kalcijem dodatno mineralizira kutikulo. Skupki kristalov apatita so v kalcijevih telescih prisotni v vseh fazah levitvenega cikla.



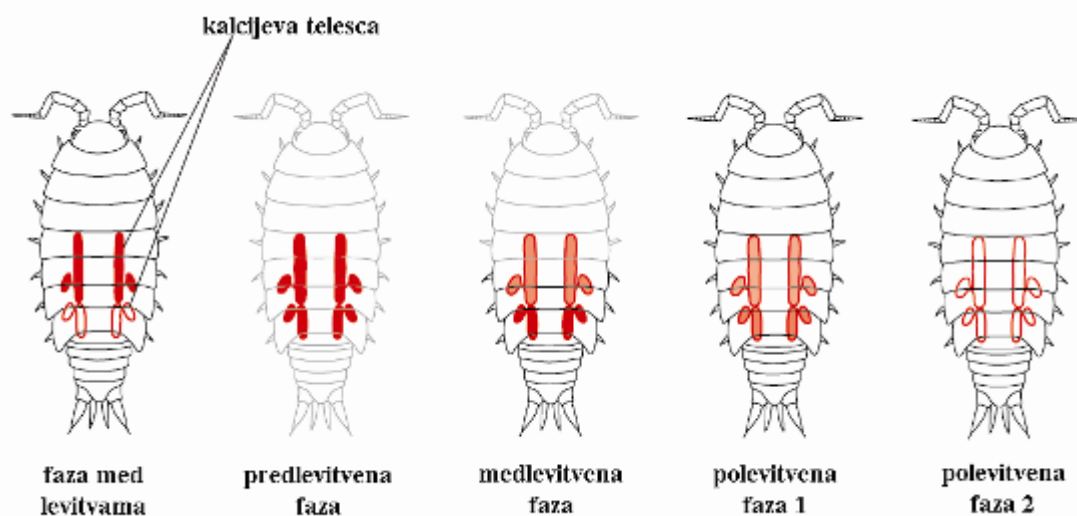
Slika 8: Dinamika kalcijevih depozitov med levitvenim ciklom jamske mokrice. Rdeče polnilo predstavlja prisotnost začasnih kalcijevih depozitov. Svetlo rdeče polnilo prikazuje potekajočo resorpcijo depozitov. Siv obris telesa predstavlja apolizo.

Figure 8: Dynamics of calcium deposits during the molt cycle in *Titanethes albus*. Red fill represents the presence of transient calcium deposits. Bright red fill represents resorption of deposits in process. Gray outlines represent apolysis.

Takšen potek dogodkov lahko povežemo s prej predstavljenim modelom, ki so ga postavili za mokrice, ki oblikujejo zgolj sternalne depozite. Pri jamski mokrici bi se v skladu s takšnim modelom kalcij iz sternalnih depozitov sprednjega dela telesa lahko porabil za mineralizacijo posterorne kutikule v medlevitveni fazi, kalcij iz kalcijevih telesc (ki ležijo v zadnjem delu telesa), pa bi se porabil za mineralizacijo anteriorne kutikule v polevitveni fazi. Seveda ni nujno, da se kalcij iz kateregakoli depozita porabi izključno za mineralizacijo kutikule v enem delu telesa. V polevitveni fazi poteka sinteza nove endokutikule v obeh delih telesa in pričakujemo lahko, da se kalcij iz kalcijevih telesc porablja za mineralizacijo endokutikule celotnega integumenta.

Pri *H. riparius* se kalcij nalaga v začasne depozite precej drugače. Sternalni depoziti se pri tej vrsti v predlevitveni fazi ne razvijejo, prisotna pa sta dva para kalcijevih telesc z

različnima vsebinama. V posteriornem paru telesc so prisotni bakterije in kalcijev fosfat, medtem ko v anteriornem paru bakterij ni, prisoten pa je zgolj kalcijev karbonat. Potek dogodkov med levitvenim ciklom pri *H. riparius* prikazuje slika 9. Steklast matriks, mineraliziran s kalcijevim karbonatom, je v anteriornih telescih prisoten že v fazi med levitvama. Vir kalcija za njegovo mineralizacijo med to fazo ni znan. Resorpcija mineralov iz stare kutikule v fazi med levitvama ni verjetna, vendar je ne moremo izključiti. Druga možnost je, da se v anteriornem paru telesc v fazi med levitvama kopičijo minerali, ki jih žival vnaša v telo s prehrano. V predlevitveni fazi se v obeh parih telesc oblikuje dodaten sloj mineraliziranega matriksa, ki je v anteriornem paru (enako kot matriks, ki je prisoten že v fazi med levitvama) mineraliziran zgolj s kalcijevim karbonatom, v posteriornem paru pa z mešanico kalcijevega karbonata in kalcijevega fosfata. V medlevitveni fazi se resorpcija kalcija začne v anteriornem paru telesc. Prvi dan po levitvi anteriornega dela telesa (polevitvena faza 1 na sliki 9) poteka resorpcija v obeh parih kalcijevih telesc, dva dneva po levitvi (polevitvena faza 2 na sliki 9) pa so zaloge v kalcijevih telescih izčrpane in resorpcija ne poteka več. Na tej točki v anteriornih telescih ni mineralov, v posteriornih telescih pa ostanejo skupki kristalov apatita, ki so prisotni skozi celoten levitveni cikel.



Slika 9: Dinamika kalcijevih depozitov med levitvenim ciklom trihoniscida *Hyloniscus riparius*. Rdeče polnilo predstavlja prisotnost začasnih kalcijevih depozitov. Svetlo rdeče polnilo prikazuje potekajočo resorpcijo depozitov. Siv obris telesa predstavlja apolizo.

Figure 9: The dynamics of calcium deposits during the molt cycle in the trichoniscid *Hyloniscus riparius*. Red fill represents the presence of transient calcium deposits. Bright red fill represents resorption of deposits in process. Gray outlines represent apolysis.

Tak potek dogodkov lahko ponovno umestimo v splošni model resorpcije in kopičenja kalcija pri mokricah, vendar so odstopanja od njega pri *H. riparius* precejšnja. V predlevitveni fazi se kalcij resorbira iz kutikule in se naloži okoli že obstoječega matriksa v

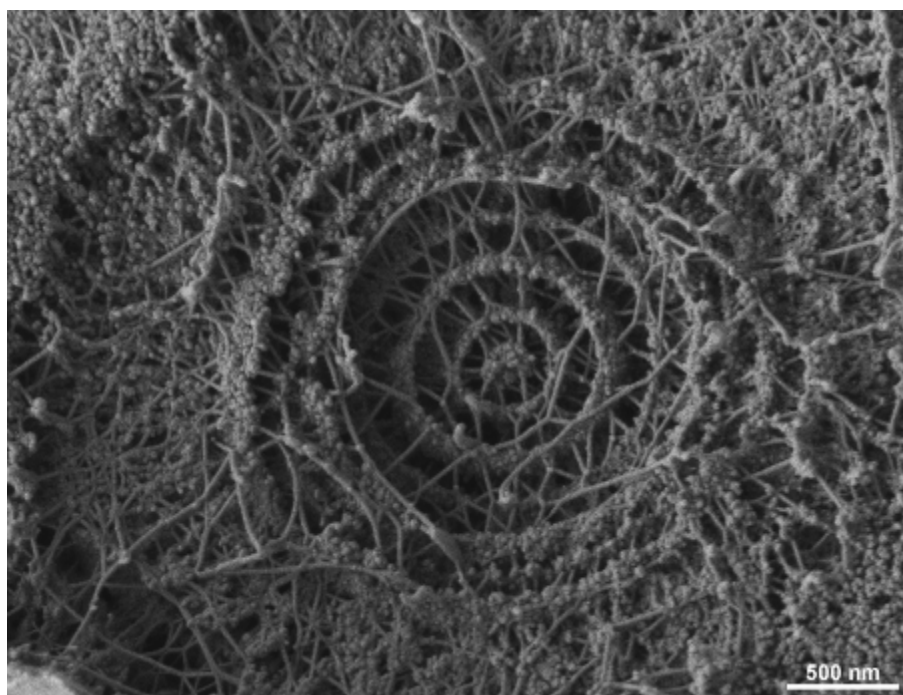
anteriornih telescih ter v steklastem sloju v posteriornih telescih. V medlevitveni fazi se kalcij resorbira iz anteriornih telesc (ki segajo v sprednji del telesa) in se porabi za mineralizacijo posteriorne kutikule. V polevitveni fazi se kalcij resorbira iz obeh parov telesc in se porabi za mineralizacijo kutikule. Pri tem gre skoraj gotovo za mineralizacijo kutikule obeh delov telesa, ker se je na začetku polevitvene faze v anteriornih telescih prisotnega še veliko mineraliziranega matriksa.

Očitna je razlika v velikosti med obema paroma kalcijevih telesc, saj so anteriorna kalcijeva telesa *H. riparius* precej večja od posteriornih. Prostornino posameznega kalcijevega telesa in njegovega stranskega izrastka lahko ocenimo, če jo računamo, kot da gre za valja. Če upoštevamo, da je povprečna debelina steklastega sloja v posteriornih telescih 10 μm , lahko ocenimo tudi prostornino steklastega sloja, če ga obravnavamo kot votel valj. Razmerje med ocenjeno prostornino anteriornih telesc v medlevitveni fazi (0,027 mm^3) in celokupno prostornino steklastega sloja posteriornih telesc v isti fazi (0,0031 mm^3) pri 4 mm veliki živali znaša okoli 8,5 : 1. Če predpostavimo, da je približno polovica minerala v steklastem sloju kalcijev fosfat, preostanek pa kalcijev karbonat, je razmerje med skladiščenim kalcijevim karbonatom in kalcijevim fosfatom približno 17 : 1. Pri do sedaj preučenih vrstah se giblje razmerje med kalcijevim karbonatom in kalcijevim fosfatom v kutikuli med 4 : 1 (*Oniscus asellus*) in 12 : 1 (*Porcellio spinicornis*; Neues in sod., 2007). Skladiščenega fosfata v steklastem sloju posteriornih kalcijevih telesc je torej glede na karbonat manj kot v tipični kutikuli mokric (Neues in sod., 2007). Taka ocena je seveda groba, ne predvideva morebitne resorpcije kalcijevega fosfata iz apatita v osrednjem delu telesc in drži le ob predpostavki, da so deleži mineralnih komponent v matriksu različnih depozitov primerljivi.

Pri jamski mokrici so pričakovana razmerja med skladiščenim kalcijevim karbonatom in kalcijevim fosfatom drugačna. Če upoštevamo, da so sternalni depoziti jamske mokrice debeli v povprečju 25 μm , steklast sloj v kalcijevih telescih pa 20 μm , predstavlja pri 12 mm veliki živali celotna prostornina sternalnih depozitov (0,054 mm^3) le okoli 20 % prostornine steklastega sloja vseh štirih kalcijevih telesc (0,28 mm^3 ; prostornina steklastega sloja je tudi v tem primeru ocenjena na enak način kot pri *H. riparius*). Če predpostavimo, da vsaj 20 % steklastega sloja predstavlja kalcijev fosfat, je razmerje med skladiščenim kalcijevim karbonatom in kalcijevim fosfatom pri tej vrsti okoli 5 : 1, kar približno ustreza razmerju med relativno količino kalcijevega karbonata in kalcijevega fosfata v kutikuli, ki znaša pri tej vrsti približno 5,5 : 1 (Hild in sod, 2009).

Zaključimo lahko, da *H. riparius* skladišči neprimerno več kalcijevega karbonata glede na kalcijev fosfat kot jamska mokrica, razmerje med obema pa je pri *H. riparius* tudi precej daleč od običajnega razmerja med kalcijevim karbonatom in kalcijevim fosfatom v kutikuli kopenskih rakov enakonožcev, medtem ko pri jamski mokrici razmerje med skladiščenimi minerali približno ustreza razmerju v kutikuli.

Ramanska spektroskopija je pokazala, da vsebujejo začasni depoziti v kalcijevih telescih zelo malo organskih komponent. Kot kaže elektronska mikroskopija, je organski matriks vendarle prisoten tako v steklastem sloju kalcijevih telesc jamske mokrice kot v anteriornih kalcijevih telescih *H. riparius* (sliki 10 in 11). V steklastem sloju kalcijevih telesc, ki vsebujejo bakterije, je matriks organiziran v koncentrične sloje in radialno usmerjena vlakna. Po organizaciji je torej podoben organskemu matriksu v sternalnih depozitih navadnega prašička (Fabritius in sod., 2005). V matriksu anteriornih telesc *H. riparius* je organizacija podobna, le da je steklasti matriks mnogo obsežnejši. Kljub navidezni podobnosti arhitekture matriksa ramanska spektroskopija kaže, da je delež organskega matriksa v sternalnih depozitih večji kot v kalcijevih telescih. To je lahko posledica večjega deleža matriksa glede na mineralne komponente v sferulah, ki sestavljajo sternalne depozite, morda pa večja količina organskega matriksa pripomore k stabilnosti ACC v sternalnih depozitih. Večja stabilnost amorfne oblike mineralov je morda potrebna zaradi težje regulacije kemijske sestave v levitvenem prostoru v primerjavi s kalcijevimi telesci, saj levitveni prostor ni v celoti obdan z epitelijem. Sternalni epitelij ob izgradnji depozitov opravlja tudi druge z levitvijo povezane funkcije, kot je sinteza nove kutikule (Ziegler, 1997). V kalcijevih telescih ta proces očitno ne poteka, saj se v predlevitveni fazi sintetizira le razmeroma tanka nova ovojnica.



Slika 10: Vrstična elektronska mikrofografija notranje površine steklastega sloja v kalcijevih telescih jamske mokrice v medlevitveni fazi. Vidno je omrežje organskega matriksa.

Figure 10: Scanning electron micrograph of the inner surface of the glassy layer within calcium bodies of *Titanethes albus* in intramolt stage. A network of organic matrix is visible.

Pri jamski mokrici se kalcij med levitvijo kopiči tako v sternalnih depozitih kot v dveh parih strukturno enakih kalcijevih telescih, medtem ko se pri *H. riparius* sternalni depoziti ne razvijejo in se kalcij kopiči zgolj v kalcijevih telescih. Prisotnost sternalnih depozitov in dveh parov enakih kalcijevih telescih, ki vsa vsebujejo bakterije, sta kot kaže splošni značilnosti trihoniscidov in rod *Hyloniscus* je v tem pogledu izjema. Druge študije navajajo sternalne depozite tudi pri rodu *Androniscus* (Ziegler, 2003), ki ima dva para enakih kalcijevih telescih (osebno opažanje), tako kot jamska mokrica. Opustitev sternalnih depozitov in njihova funkcionalna nadomestitev z anteriornimi kalcijevimi telesci je najverjetneje izpeljana značilnost, ki se je razvila (med preučeni trihoniscidi) le pri rodu *Hyloniscus*.



Slika 11: Presevna elektronska mikrografija matriksa v anteriornem kalcijevem telescu *Hyloniscus riparius* medlevitveni fazi. Vidno je omrežje vlaken. Osrednji del matriksa je na levi strani slike. Skrajno desno je viden epitelij kalcijevega telesca.

Figure 11: Transmission electron micrograph of the matrix within an anterior calcium body of *Hyloniscus riparius* in intramolt stage. A network of fibers is visible. The center of the matrix is to the left of the image. The calcium body epithelium is visible to the right.

Resorpcija sternalnih depozitov, ki vsebujejo predvsem CaCO_3 , poteka pri jamski mokrici v medlevitveni fazi. Pri *H. riparius* poteka v tej fazi predvsem resorpcija mineralov iz anteriornih kalcijevih telescih, ki vsebujejo zgolj CaCO_3 . Na podlagi tega lahko zaključimo, da se v medlevitveni fazi kalcijev karbonat resorbira v večji meri kot kalcijev fosfat. Z vidika mineralizacije kutikule je to lahko funkcionalno. Po podatkih Hild in sod. (2008) je distalna eksokutikula pri mokricah mineralizirana s kalcitom, medtem ko so proksimalni deli kutikule mineralizirani z ACC, ki je stabiliziran s kalcijevim fosfatom. V kolikor predpostavimo, da se eksokutikula mineralizira najprej, je smiselno, da se iz začasnih depozitov med mineralizacijo eksokutikule resorbira pretežno CaCO_3 , med mineralizacijo endokutikule pa tako kalcijev fosfat kot kalcijev karbonat. Predpostavljeno časovno zaporedje mineralizacije posameznih slojev kutikule lahko podpremo s podatki iz literature. Raziskava ultrastrukture integumenta s precipitacijo kalijevega antimonata pri deseteronožcih (Compère in sod., 1993) ter študija integumenta navadnega prašička z vrstično elektronsko mikroskopijo in ramansko spektroskopijo (Neues in sod., 2011) kažeta, da se eksokutikula v veliki meri mineralizira takoj po levitvi, v endokutikulo pa se minerali nalagajo sočasno z njeno sintezo. Neues in sod. (2011) žal ne navajajo podatkov o morebitni mineraliziranosti nove eksokutikule pred levitvijo, zato pri mokricah ni izključena.

Če upoštevamo, da endokutikula ob CaCO_3 vsebuje tudi manjhen delež kalcijevega fosfata, je z vidika skladiščenja mineralov med levitvijo kopičenje kalcijevega fosfata v začasnih depozitih kalcija smiselno. Takšno skladiščenje je bilo predlagano tudi pri nekaterih drugih rakih. Greenaway in Farrelly (1991) sta predlagala, da služijo velike zunajcelične granule pri rakovici *Holthuisana transversa* shranjevanju fosforja med levitvijo. Granule, ki vsebujejo veliko kalcija in fosforja, so opazili tudi v hepatopankreasu rakovice *Callinectes sapidus* v fazi med levitvama, pri čemer bi vezani fosfor lahko pripomogel k mineralizaciji kutikule (Becker in sod., 1974).

Epiteliji kalcijevih telesc jamske mokrice in posteriornih kalcijevih telesc *H. riparius* kažejo v vseh fazah levitvenega cikla značilnosti transportnih epitelijev. Te so npr. številni mitohondriji, izrazit bazalni labirint in nagubana apikalna plazmalema (Cioffi, 1984). Takšne so značilnosti celic epitelija črevesa in Malpighijevih cevok nekaterih členonožcev (Cioffi, 1984) ter celic vretenčarskih epitelijev, ki intenzivno transportirajo protone in hidrogenkarbonatne ione (Brown in Breton, 1996). V osmoregulacijskem tkivu škrk kopenske rakovice *Gecarcinus lateralis* je apikalna površina celic nekoliko nagubana, izrazite invaginacije bazolateralne membrane pa segajo vse do apikalnega dela celic (Copeland, 1968). V kalcijevih telescih v fazi med levitvama ultrastrukturne značilnosti, povezane s transportom skozi epitelij, niso tako izrazite kot v navedenih primerih, so jim pa bolj podobne v polevitveni fazi. Za razliko od hepatopankreasa mokric (Žnidaršič in sod., 2003) epiteljske celice kalcijevih telesc nimajo enakomerno razporejenih mikrovilov, temveč precej neurejeno porazdeljene prstaste izrastke in invaginacije.

Pri *H. riparius* so epiteliji posteriornih in anteriornih kalcijevih telesc ultrastrukturno podobni. Najbolj očitna razlika je, da epitelij anteriornih kalcijevih telesc v fazi med levitvama nima izrazito nagubane apikalne površine, medtem ko ima epitelij posteriornih kalcijevih telesc nagubano apikalno plazmalemo v vseh fazah levitvenega cikla. Nagubanost apikalne površine v fazi med levitvama lahko zato povežemo s prisotnostjo bakterij v kalcijevih telescih. Če povečana površina plazmaleme epiteljskih celic nakazuje aktivne transportne procese, to pomeni, da v kalcijevih telescih z bakterijami intenziven transport skozi epitelij poteka med celotnim levitvenim ciklom. Če so bakterije metabolno aktivne, porabljajo hranila in sproščajo produkte metabolizma v okolje. Nagubanost plazmaleme epitelija kalcijevih telesc z bakterijami je zato lahko povezana z dovajanjem hranil in odvajanjem produktov metabolizma bakterijske populacije v njih.

V citoplazmi epiteljskih celic vseh preučeni kalcijevih telesc so prisotne glikogenske granule. Te so lahko zaloga energije (Brown in Ransom, 2007) za intenziven metabolizem epiteljskih celic, kar potrjuje tudi prisotnost številnih mitohondrijev v njihovi citoplazmi. Glikogen bi lahko predstavljal tudi vir ogljika za sintezo organskega matriksa (Graf in Meyran, 1985).

Ultrastrukturalne značilnosti epitelija kalcijevih telesc med resorpcijo kalcija pri *H. riparius* so zelo podobne opisanim značilnostim sternalnega epitelija med resorpcijo sternalnih depozitov pri drugih mokricah (Ziegler, 1996; Glötzner in Ziegler, 2000).

Na podlagi predhodnih raziskav posteriornih cekov postranic (Graf in Meyran, 1985), integumenta deseteronožcev (Greenaway in Farrelly, 1991) in sternalnega epitelija enakonožcev (Ziegler, 1996; Glötzner in Ziegler, 2000) ter na podlagi rezultatov naših raziskav epitelijev kalcijevih telesc *H. riparius* lahko ugotovimo, da kažejo epiteliji mokric med resorpcijo začasnih depozitov kalcija, ko poteka transport kalcija v apiko-bazalni smeri, več skupnih ultrastrukturnih značilnosti. Te so:

- močno nagubana apikalna plazmalema;
- omrežje povečanih medceličnih prostorov med sosednjimi celicami;
- elektronsko goste zunajcelične granule nanometrskih dimenzij, ki se oblikujejo na pedicelih bazolateralne plazmaleme v medceličnih prostorih in pri preučeni vrsti vsebujejo kalcij in fosfor.

Med resorpcijo kalcija iz kutikule ali začasnih depozitov je koncentracija Ca^{2+} v hemolimfi verjetno nižja kot v mineraliziranem matriksu, zato bi lahko transport skozi epitelij potekal pasivno. Več argumentov govori v prid transcelularnemu kot paracelularnemu transportu. Povečanje relativne prostornine mitohondrijev nakazuje na povečano intenzivnost oksidativnega energijskega metabolizma (Glötzner in Ziegler, 2000), povečanje površine apikalne in bazolateralne plazmaleme pa kaže na intenzivnejši transport skozi plazmalemo. Povišanje celokupne koncentracije kalcija v citoplazmi in koncentriranje kalcija v endoplazemskem retikulumu (Ziegler, 2002) ter tvorba granul, ki vsebujejo kalcij (Ziegler, 1996; Graf in Meyran, 1985), kažejo na privzem kalcija v celice in njegovo izločanje v vezani obliki. Vezava kalcija bi lahko ohranjala nizko lokalno koncentracijo prostih Ca^{2+} ionov in s tem omogočala ohranjanje visokega koncentracijskega gradienta kalcija med kalcijevim depozitom in medceličnino (Graf in Meyran, 1985; Ziegler in sod., 2005).

Nekatere od značilnosti epitelijev med resorpcijo depozitov kalcija, npr. povečanje površine plazmaleme in relativne prostornine mitohondrijev, lahko povežemo tudi s transportom hidrogenkarbonatnih ionov, ki se transportirajo hkrati s kalcijem. Vprašanje transporta kalcija skozi epitelij zato ni dokončno razloženo. Dodatne podatke bi lahko dalo spremljanje spreminjanja znotrajcelične koncentracije Ca^{2+} *in vivo*. Večina trenutno razpoložljivih podatkov o transportu kalcija skozi epitelije pri rakih je omejena na ultrastrukturne značilnosti celic, poznavanje mehanizmov kalcijevega transporta na celičnem nivoju pa je še vedno zelo omejeno. Kot kaže so ultrastrukturne spremembe epitelijev izrazitejše med transportom od apikalne proti bazalni strani epitelija med resorpcijo depozitov kot med transportom v nasprotni smeri med njihovo mineralizacijo.

Elektronsko goste granule v medceličnih prostorih so bile med transportom kalcija v apikalno-bazalni smeri opažene pri različnih vrstah rakov tako v epitelijskih začasnih depozitih kalcija kot v epidermu (Graf in Meyran, 1985; Greenaway in Farrelly, 1991; Ziegler, 1996, 1997a). Pri preučeni vrsti je bilo ugotovljeno, da vsebujejo granule kalcij in fosfor (Greenaway in Farrelly, 1991; Ziegler 1996, 2002). Kaže, da kalcificirane granule pri različnih skupinah rakov nastajajo na izvihkih plazmaleme (pedicelih). Elektronsko gosta območja na lateralnih membranah, ki so vidna pri *H. riparius*, lahko pomenijo, da se na območjih, kjer nastajajo granule, koncentrirajo membranski proteini.

Difuzija Ca^{2+} v citoplazmi je počasna. Če granule med celicami nastajajo tik pod subapikalnimi celičnimi stiki (Graf in Meyran, 1985), do koder segajo invaginacije apikalne površine, bi se med morebitnim transcelularnim transportom koncentracija Ca^{2+} lahko dvignila tudi samo v skrajno apikalnem delu citoplazme oz. v izvihkih plazmaleme, v večjem delu citoplazme pa ostala nespremenjena. Nenavadno je, da so granule v medceličnih prostorih epitelijskih anteriornih kalcijevih telesc *H. riparius* v polevitveni fazi v bazalnih delih epitelijskih drobnejše in povezane z membranskimi pediceli, bolj proti sredini epitelijskih pa večje in proste. Po analogiji z granulami v posteriornih cekih postranic (Graf in Meyran, 1985) bi to lahko razložili kot nastajanje granul v bazalnem delu celic in njihovo rast ter sproščanje proti apikalnemu delu. To je nenavadno, saj transporta granul v tej smeri med resorpcijo depozitov ne pričakujemo. Podobno razporeditev granul sta opazila Glötzner in Ziegler (2000) v sternalnem epiteliju pri nekaterih predstavnikih družine Ligidae, vendar ga v svojem delu nista pojasnila. V primeru anteriornih kalcijevih telesc *H. riparius* je lahko nastajanje granul v bazalnem delu celic tudi posledica zelo globokih invaginacij apikalne plazmaleme, ki segajo preko polovice epitelijske in tako omogočajo transport ionov v bazalnem delu celic.

Ob transportni funkciji opravljajo epitelijski, ki izgrajujejo začasne depozite kalcija, tudi sekrecijsko funkcijo. V primeru gastrolitov morajo izločiti vse organske komponente gastrolita in novo kutikulo želodca (Travis, 1960), v primeru sternalnih depozitov pa izločijo organski matriks le-teh in novo sternalno kutikulo (Ziegler, 1996). Ker se nova epikutikula sintetizira že zgodaj v predlevitveni fazi (Ziegler, 1996), pozneje v predlevitveni fazi pa se sintetizira tudi že nova eksokutikula, lahko zaključimo, da plazmalema epitelijskih celic ne more biti v neposrednem stiku z nastajajočimi sternalnimi depoziti. Podobno lahko zaključimo za izgradnjo matriksa v kalcijevih telescih. Ker se v predlevitveni fazi nove plasti matriksa oblikujejo v prostoru med staro in novo ovojnico in je nova ovojnica med nastajanjem teh plasti že prisotna (epitelij kalcijevih telesc jo sintetizira v zgodnji predlevitveni fazi, glavna steklastega sloja pa nastane v pozni predlevitveni fazi), tudi v tem primeru epitelijske celice niso v neposrednem stiku z rastočim mineraliziranim matriksom, tako kot so med sintezo in mineralizacijo kutikule ali med sintezo ovojnice v kalcijevih telescih. Če celice niso v neposrednem stiku z nastajajočim matriksom, ne morejo neposredno usmerjati njegove organizacije.

Mineraliziran matriks v kalcijevih telescih zato najverjetneje nastaja s samoorganizacijo makromolekul in mineralnih soli v tekočem mediju.

Stare ovojnice so v kalcijevih telescih očitno pomembne pri izgradnji mineraliziranega matriksa, saj se nastajanje steklastega sloja pri jamski mokrici začne z oblikovanjem sferul na površini stare ovojnice, ki služi kot osnova za nalaganje mineraliziranega matriksa. Lamine lahko služijo kot ogrodje za začetek izgradnje matriksa, ker predstavljajo trdno maso imobiliziranih makromolekul, na katere se lahko začnejo nalagati kalcijeve soli (Saito in sod., 1997; Naka, 2007) in so hkrati lahko osnova za vezavo in nalaganje organskih komponent matriksa. Prav tako predstavlja stara ovojnica notranjo površino steklastega sloja v posteriornih kalcijevih telescih pri *H. riparius*, zato lahko predpostavimo, da se sloj v tem primeru oblikuje na podoben način. Podobno se kontinuiran sloj matriksa v anteriornih telescih pri *H. riparius* oblikuje okoli najbolj zunanje lamine (verjetno stare ovojnice iz predhodnega levitvenega cikla). Pri *H. riparius* nastajajo majhne sferule tudi na drugih, bolj notranjih laminah v obeh parih kalcijevih telescih. Prav tako so pri jamski mokrici ter pri *H. riparius* ob laminah v kalcijevih telescih kristali apatita bolj koncentrirani.

Zanimivo je, da je tudi nastanek drugih začasnih depozitov kalcija pri rakah običajno vezan na že obstoječe zunajcelične matrikse, zlasti kutikularne strukture. Tako npr. gastroliti nastajajo med epitelijem gastrolitnega diska in staro kutikulo želodca (Travis, 1960), sternalni depoziti pa med sternalnim epitelijem in staro integumentalno kutikulo (Fabritius in Ziegler, 2003). Model nastanka gastrolitov predvideva, da nastajajo od kutikule proti epiteliju, kar pomeni, da predstavlja stara kutikula osnovo za začetek njihove izgradnje. Ker so tudi v primeru sternalnih depozitov celice fizično ločene od nastajajočih sternalnih depozitov z novo kutikulo, lahko predpostavimo, da nastajanje sternalnih depozitov poteka preko samoorganizacije. Ta predpostavka je v soglasju z modelom nastanka sternalnih depozitov, ki sta ga postavila Fabritius in Ziegler (2003).

3.3 RAZLIKE V SKLADIŠČENJU KALCIJA MED POVRŠINSKIMI IN JAMSKIMI MOKRICAMI

Pri veliki jamski mokrici se med resorpcijo sternalnih depozitov medcelični prostori anteriornega sternalnega epitelija sicer povečajo, vendar v njih nismo opazili elektronsko gostih granul (Priloga B), ki so jih opisali pri drugih mokricah (Ziegler, 1997; Glötzner in Ziegler, 2000). To velja tudi za epitelij kalcijevih telescih jamske mokrice. Granule v medceličnih prostorih se pri tej vrsti med transportom kalcija skozi epitelij bodisi ne pojavijo ali pa so zelo redke. Odsotnost granul med transportom kalcija lahko odraža manjšo intenzivnost transporta skozi epitelij pri jamski mokrici v primerjavi s površinskimi sorodniki.

Glötzner in Ziegler (2000) sta ugotovila, da so granule v medceličnih prostorih sternalnega epitelijske pri nekaterih vrstah mokric prisotne tako v predlevitveni kot v medlevitveni fazi. Morda granule odražajo veliko intenzivnost transporta kalcija ne glede na njegovo smer. Ker je izgradnja depozitov kalcija praviloma počasnejša kot njihova resorpcija, v tem primeru pri nekaterih vrstah v času predlevitvene faze granule niso prisotne, pojavijo pa se v medlevitveni fazi, ko se transport kalcija pospeši.

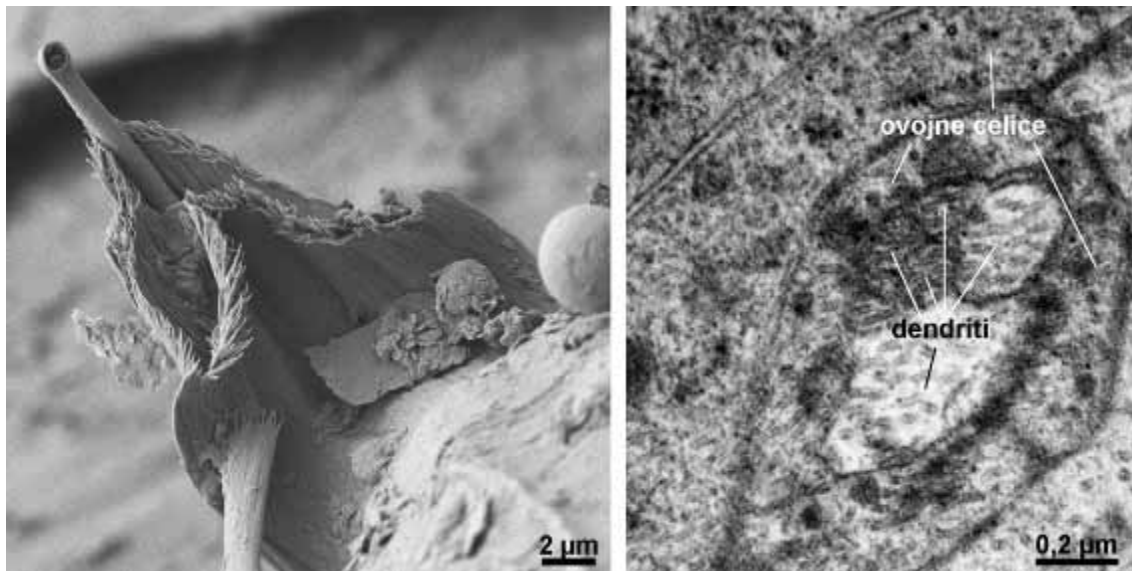
Ramanska spektroskopija je pokazala, da vsebujejo sternalni depoziti jamske mokrice nekoliko večji delež kalcijevega fosfata v primerjavi s sternalnimi depoziti navadnega prašička (Hild in sod., 2008). Če to povežemo s kopičenjem tako kalcijevega fosfata kot kalcijevega karbonata v kalcijevih telescih, lahko zaključimo, da jamska mokrica v primerjavi s površinskimi mokricami med levitvijo skladišči več fosforja, morda zaradi manjše dostopnosti tega elementa v jamskem okolju. Če upoštevamo, da kalcijev fosfat stabilizira ACC (Raz in sod., 2002), lahko pomeni večja količina fosfatnih ionov v sternalnih depozitih tudi večjo stabilnost amorfnih mineralov, ki je morda povezana z razmeroma dolgo predlevitveno fazo jamske mokrice ali morebitno večjo prepustnostjo kutikule in posledično slabšim nadzorom nad kemijsko sestavo levitvenega prostora pri jamski mokrici.

Sternalni depoziti jamske mokrice so v celoti sestavljeni iz drobnih sferul. Pri nobenem od pregledanih osebkov v sternalnih depozitih nismo opazili proksimalnega homogenega sloja mineraliziranega matriksa, kakršen je prisoten pri nekaterih drugih mokricah (Ziegler in Miller, 1997). Prav tako ta sloj do sedaj ni bil opažen pri predstavnikih družine Ligiidae. Njegova odsotnost pri jamski mokrici je lahko prilagoditev te mokrice na podzemeljsko okolje, lahko pa skupna značilnost predstavnikov skupine Synocheta, kar bi lahko preverili z analizo sternalnih depozitov pri bližnjih sorodnikih jamske mokrice. Zanesljivih podatkov o zgradbi sternalnih depozitov drugih trihoniscidov trenutno ni na voljo. Povezavo med filogenijo in zgradbo sternalnih depozitov sta predlagala Ziegler in Miller (1997). Zanimivo je, da so sferule, ki sestavljajo sternalne depozite jamske mokrice (tipično 0,3 μm v premeru), nekoliko manjše kot pri površinskih vrstah (od 0,3 μm do 1,2 μm v premeru pri večini vrst; Ziegler in Miller, 1997).

3.4 ŠČETINASTI ŽLEZNI ORGANI

Ščetinasti žlezni organi samcev jamske mokrice četrtega pleonita so kompleksne kutikularne strukture s številnimi porami, povezanimi s specializiranimi žlezami, ki jih samice nimajo. Iz apeksa tuberklov na četrtem pleonitu izraščajo epikutikularne ščetine in na njih so koncentrirane senzile, medtem ko so stranska pobočja tuberklov pokrita s ploskimi epikutikularnimi luskami.

Z gotovostjo lahko trdimo, da so senzile na tuberklih funkcionalno specializirane in drugačne od senzil drugje na telesu. Senzile na tuberklih namreč vsebujejo le tri dendrite, senzile na drugih delih telesa jamske mokrice (slika 12) pa po zgradbi približno ustrezajo senzilam s sedmimi dendriti, ki jih je na antenah jamske mokrice opisal Crouaou (1994). Nobeden od dendritov senzil na tuberklih ni podoben mehanosenzoričnemu dendritu v senzilah anten (Crouaou, 1994), zato lahko predpostavimo, da so senzile na ščetinastih žleznih organih jamske mokrice najverjetneje kemosenzorične. Prisotnost por na senzilah ne govori nujno v prid kemosenzorični funkciji, saj lahko pora, ki je prisotna terminalno na senzili, omogoča tudi dendritsko povezavo do stare kutikule med predlevitveno fazo in s tem ohranja senzorično funkcionalnost starih senzil med pripravo na levitev (Kouyama in Shimozawa, 1984). Za prepričljive zaključke glede funkcije senzil bi bili potrebni fiziološki poskusi.



Slika 12: Senzile na površini tergитov jamske mokrice. Levo vrstična elektronska mikrografija senzile, desno prežez senzile v nivoju epiderma. Vidnih je več dendritov in ovojnih celic.

Figure 12: The sensilla on the surface of tergites in *Titanethes albus*. Left: a scanning electron micrograph of a sensillum. Right: section through a sensillum at the level of the epidermis. Several dendrites and enveloping cells are visible.

Samčeve žleze so pri jamski mokrici zelo številčne in zapolnjujejo večji del pleona in uropodov. Zanimivo je, da samčeve žleze ne izločajo zgolj na površino tuberklov na pleonitu 4, temveč so na pleonu prisotne tudi druge sekrecijske površine, na katerih se koncentrirajo izvodila žlez, značilnih le za samce. Takšne površine se nahajajo anteriorno na pleonitu 4, lateralno in ob anteriornem robu pleonita 5 ter anteriorno na pleotelzonu. Samčeve žleze jamske mokrice nimajo enotne ultrastrukture, temveč so v pleonu samcev prisotni trije tipi žlez, ki izločajo na različnih površinah:

- žleze tipa 1 se nahajajo v pleonitih 2-5 in izločajo skozi tuberkle pleonita 4 ter skozi sekrecijske površine lateralno na pleonitu 5;
- žleze tipa 2 se nahajajo v eksopoditih uropodov in izločajo na njihovo površino;
- žleze tipa 3 se nahajajo mediano v pleonitu 5 in v pleotelzonu ter izločajo skozi sekrecijske površine ob anteriornih robovih pleonita 5 in pleotelzona.

Ultrastrukturno najbolj izstopajo žleze tipa 1, ki so tudi edini tip žlez, ki izloča skozi pore na tuberklih pleonita 4. Žleze tipa 1 so žleze rozetastega tipa, ki jih sestavlja 3-5 sekrecijskih celic, osrednja celica ter kanalska celica. Podrobnosti njihove zgradbe ne ustrezajo opisom rozetastih žlez drugih rakov (Johnson in Talbot, 1987; Talbot in sod., 1991). Običajno oblikujejo sekrecijske celice ugreznitve plazmaleme, po katerih se stekajo sekrecijski produkti v osrednji kanal, ki teče skozi osrednjo celico ter se nadaljuje v kanal kanalske celice (Talbot in Demers, 1993). Pri samčevih žlezah tipa 1 jamske mokrice je osrednja celica zvezdaste oblike, njeni izrastki pa se zajedajo globoko v invaginacije sekrecijskih celic. Med membrano izrastka ter membrano sekrecijske celice tako nastane kanalček, zatesnjen s celičnimi stiki. Kanalček se odpira v kanal osrednje celice, ki se nadaljuje v izvodilo kanalske celice. Način nastanka kanalov med izrastki osrednje celice in invaginacijami sekrecijske celice spominja na krpate žleze, ki so prav tako prisotne pri kopenskih enakonožcih in imajo najverjetneje obrambno funkcijo (Weirich in Ziegler, 1997). Krpate žleze so trocelične; prisotna je ena dvojedrna sekrecijska celica, ki jo s citoplazemskimi izrastki objema vmesna celica, do površine pa vodi kanalska celica. Pri krpatih žlezah kanalčki, ki vodijo do sekrecijske produkta v kanal osrednje celice, nastanejo med plazmalemo sekrecijske celice in izrastkom vmesne celice, vendar ti izrastki niso izrazito ugreznjeni v sekrecijsko celico kot pri samčevih žlezah jamske mokrice, temveč ležijo na njeni površini.

Sekrecijski produkti samčevih žlez tipa 1 so najverjetneje proteinski. Ohranijo se ob fiksaciji z aldehidi, so elektronsko gosti, v izvodilih žlez se ohranijo v obliki kapelj in se kopičijo na površini tuberklov, na njihovo proteinsko naravo pa kaže tudi obarvanje z barvili eozinom, Ponceau 2S in Coomassie modrim (Priloga C).

Druga dva tipa samčevih žlez jamske mokrice po zgradbi ustrezata rozetastim tegumentalnim žlezam drugih rakov (Talbot in Demers, 1993). Oba tipa se razlikujeta glede na kemijsko naravo sekrecijskih produktov, saj so sekrecijska zrna v žlezah tipa 2 s presevno elektronsko mikroskopijo videti elektronsko gosta, medtem ko so sekrecijska zrna v žlezah tipa 3 videti elektronsko redka. Sekrecijske celice teh dveh tipov žlez same oblikujejo ugreznitve membrane za odvajanje sekrecijskih produktov in za razliko od sekrecijskih celic v žlezah tipa 1 vsebujejo zelo obsežen Golgijev aparat.

Žleze, povezane s ščetinastimi žleznimi organi so pri jamski mokrici precej drugačne kot pri vrsti *Trichoniscus alexandrae*, ki je prav tako jamski trihoniscid (Lombardo in sod.,

2006). Za razliko od jamske mokrice pri *T. alexandrae* skozi ščetinaste žlezne organe izločajo trocelične žleze. Zelo verjetno so se ščetinasti žlezni organi obeh vrst razvili neodvisno. Vzporednica med žlezami obeh vrst je, da so tudi pri *T. alexandrae* sekrecijska zrna v velikih sekrecijskih celicah samčevih žlez elektronsko gosta (zanimivo je, da je pri tej vrsti tudi vmesna celica sekrecijska in vsebuje elektronsko svetla zrna). Tako kot pri jamski mokrici so tudi pri *T. alexandrae* v bližini izvodil žlez prisotne senzile. Te so zgrajene nekoliko drugače kot senzile na tuberklih samcev jamske mokrice, ker so oživčene s šestimi dendriti. Ali gre tudi v tem primeru za specializirane senzile, ni jasno. Tudi v senzilah na ščetinastih žleznih organih *T. alexandrae* nobeden od dendritov ne ustreza domnevno mehanosenzoričnemu dendritu, kakršen je prisoten v senzilah na antenah jamske mokrice (Crouau, 1994), zato predpostavljajo, da so kemosenzorične (Lombardo in sod., 2006).

Pridobljeni podatki o ultrastrukturi sekrecijskih celic in naravi sekrecijskih produktov ščetinastih žleznih organov ne podpirajo uveljavljene hipoteze o njihovi funkciji, ki predvideva, da izločajo hlapne feromone. Kot kaže, izločajo ščetinasti žlezni organi visokomolekularne, najverjetneje proteinske sekrecijske produkte, ki se zadržujejo na površni telesa in morda delujejo kot kontaktni feromoni.

3.5 SKLEPI

Celični procesi, povezani z levitvijo, so pri jamski mokrici podobni, a počasnejši kot pri površinskih mokricah. Za razliko od preučenihi površinskih vrst pri jamski mokrici po levitvi ne pride do modifikacij epikutikule.

Pri obeh preučenihi vrstah mokric so prisotni začasni depoziti kalcija. Tako pri jamski mokrici kot pri *H. riparius* so prisotna kalcijeva telesca, pri jamski mokrici pa tudi sternalni depoziti. Ti se pri *H. riparius* ne pojavijo, kar je med kopenskimi enakonožci redkost. Kalcijeva telesca so epiteljske vrečke, ki ležijo ventrolateralno v glavni telesni votlini ob prebavilu. Pri jamski mokrici so vsa kalcijeva telesca zapolnjena z bakterijami in vsebujejo okoli 100 nm velike kristale apatita. Pri *H. riparius* so bakterije in apatit prisotni zgolj v posteriornem paru kalcijevih telesc, v anteriornem paru pa bakterij ni in matriks v njih je mineraliziran s kalcijevim karbonatom.

Arhitektura in sestava matriksa kalcijevih telesc se v levitvenem ciklu spreminjata. V predlevitveni fazi epiteliji kalcijevih telesc izločijo tanko zunajcelično ovojnico, ki obdaja material v lumnu organa. V kalcijevih telescihi jamske mokrice ter posteriornih kalcijevih telescihi *H. riparius* se med staro in novo ovojnico razvije steklast sloj matriksa, ki je mineraliziran s kalcijevim karbonatom ter kalcijevim fosfatom. V anteriornih kalcijevih telescihi *H. riparius* se naloži dodaten matriks, mineraliziran s kalcijevim karbonatom.

Steklast sloj v kalcijevih telescih jamske mokrice ter v posteriornih kalcijevih telescih *H. riparius* se po levitvi razgradi. Prav tako se po levitvi popolnoma resorbira kalcijev karbonat v anteriornih kalcijevih telescih *H. riparius*.

V levitvenem ciklu se spreminja tudi ultrastruktura epitelijev kalcijevih telesc. Med resorpcijo mineralov je epitelij kalcijevih telesc pri *H. riparius* ultrastrukturno podoben transportnim epitelijem med resorpcijo kalcijevih depozitov pri drugih rakih. Apikalna površina se močno poveča z gubanjem, medcelični prostori med sosednjimi celicami se razširijo in v njih se pojavijo nekaj deset nanometrov velike elektronsko goste granule, ki nastajajo na kratkih izrastkih lateralne plazmaleme epiteljskih celic. Takšne granule se pojavijo v polevitveni fazi tudi v posteriornih kalcijevih telescih, nismo jih pa opazili pri jamski mokrici. Apikalna površina kalcijevih telesc, ki vsebujejo bakterije, je nagubana skozi celoten levitveni cikel.

V kopičenju in transportu kalcija so med površinskimi in jamskimi vrstami prisotne razlike, vendar ne moremo z gotovostjo trditi, da gre za prilagoditve na jamsko okolje. Če razmerje med količinami posameznih mineralov v začasnih depozitih kalcija med vrstama primerjamo, kopiči jamska mokrica relativno več kalcijevega fosfata glede na kalcijev karbonat v primerjavi s *H. riparius*. Za razliko od predstavnikov skupine Crinocheta so sternalni depoziti jamske mokrice sestavljeni z golj iz sferul, ki so manjše kot pri preučeni površinski mokricah. Med transportom kalcija skozi epitelij kalcijevih telesc jamske mokrice se ne pojavijo elektronsko goste granule, ki so pri *H. riparius* prisotne. Odsotnost elektronsko gostih granul lahko odraža manj intenziven transport kalcija skozi epitelij pri jamski mokrici.

Potrdili smo, da ščetinasti žlezni organi samcev jamske mokrice vsebujejo rozetaste eksokrine žleze, na organih pa so ob raznolikih epikutikularnih strukturah prisotne tudi specializirane senzile. Žleze ščetinastih žleznih organov jamske mokrice so po zgradbi edinstvene in niso podobne samčevim žlezam rodu *Trichoniscus*, kar nakazuje na neodvisen razvoj teh organov pri različnih rodovih trihoniscidov. Žleze v ščetinastih žleznih organih najverjetneje izločajo peptide. Drugačne rozetaste žleze, značilne le za samce, so prisotne tudi v drugih delih pleona samcev jamske mokrice.

4. POVZETEK (SUMMARY)

4.1 POVZETEK

Značilnost rakov (Crustacea) je kalcificirano zunanje ogrodje (kutikula), ki ga med rastjo, razmnoževanjem in razvojem redno nadomeščajo z levitvijo. Pri številnih skupinah rakov so se razvili različni začasni depoziti kalcija, v katerih se kalcij običajno kopiči v obliki amorfnega kalcijevega karbonata. Ti depoziti se običajno pojavijo zgolj v določenih fazah levitvenega cikla in verjetno zmanjšujejo odvisnost rakov od okoljskih virov kalcija. Sintezo ter mineralizacijo kutikule in mineraliziranih zunajceličnih matriksov začasnih depozitov kalcija opravljajo različni sekrecijski epiteliji integumenta in prebavila.

Pri kopenskih enakonožcih (Oniscidea) se kalcij v predlevitveni fazi levitvenega cikla običajno kopiči v obliki sternalnih depozitov CaCO_3 . Sternalni depoziti se pojavijo v levitvenem prostoru med staro in novo kutikulo sprednjih štirih sternitov pereona. Družina Trichoniscidae je razmeroma slabo preučena skupina kopenskih enakonožcev z mnogimi jamskimi predstavniki. Pri trihoniscidih so prisotna kalcijeva telesa, posebni organi, v katerih se kopiči kalcij. Proces levitve in spreminjanje strukture in sestave začasnih depozitov kalcija med levitvenim ciklom pri trihoniscidih še niso bili preučeni. Pri samcih nekaterih trihoniscidov so prisotni ščetinasti žlezni organi, na zunaj vidni kot porozne površine, prekrte z raznolikimi kutikularnimi strukturami. Struktura teh organov je bila doslej raziskana le pri eni vrsti, njihova funkcija pa ostaja neznana.

V naši raziskavi smo spremljali levitev podzemeljskega trihoniscida velike jamske mokrice *Titanethes albus* (Koch) v laboratoriju in preučili ultrastrukturo njenega integumenta med levitvenim ciklom. Pri tej vrsti smo podrobno preučili tudi zgradbo ščetinastih žleznih organov in za samce značilnih eksokrinih žlez. Opisali smo arhitekturo in sestavo kalcijevih telesc in njihovo dinamiko med levitvenim ciklom. Poleg jamske mokrice smo v raziskavo vključili tudi trihoniscida *Hyloniscus riparius* (Koch), ki živi v listni stelji. Tudi pri tej vrsti smo opisali zgradbo kalcijevih telesc ter spremembe njihove zgradbe med levitvenim ciklom.

Osebke jamske mokrice in *H. riparius* smo vzdrževali v laboratoriju. Živali v različnih fazah levitvenega cikla smo fiksirali v 4% formaldehidu in pripravili parafinske rezine. Druge osebke smo fiksirali v mešanici paraformaldehida in glutaraldehida v kakodilatnem pufu, postfiksirali z osmijevim tetroksidom in vklopili v sintetično smolo za pripravo poltankih in ultratankih rezin. Enako fiksirane vzorce smo tudi posušili v heksametildisilazanu (HMDS), pritrdili na nosilce, naprašili s platino in z vrstično elektronsko mikroskopijo preučili zunanjo morfologijo ščetinastih žleznih organov.

Na rezinah smo s svetlobno mikroskopijo ter presežno elektronsko mikroskopijo preučili

histološke in ultrastrukturne značilnosti kalcijevih telesc v levitvenem ciklu ter ščetinastih žleznih organov pri živalih v fazi med levitvama. Iz serijskih parafinskih rezin smo izdelali 3D rekonstrukcijo prostorske razporeditve eksokrinih žlez v pleonu samcev jamske mokrice. Prisotnost bakterij v tkivih jamske mokrice smo potrdili s hibridizacijo *in situ* na histoloških rezinah s fluorescentno nukleotidno sondo, specifično za evbakterijsko ribosomsko RNA.

Kalcijeva telesca v različnih fazah levitvenega cikla smo izolirali v metanolu in posušili na zraku. Tako pripravljene vzorce smo pritrdili na nosilce, zlomili, da smo izpostavili njihovo notranjost, jih naprašili s platino in opazovali z vrstičnim elektronskim mikroskopom. Enako smo pripravili tudi sternalne depozite jamske mokrice. Nekatero vzorce kalcijevih telesc jamske mokrice smo naprašili z ogljikom in analizirali njihovo elementno sestavo z energijsko-disperzijsko rentgensko spektrometrijo (EDXS) v vrstičnem elektronskem mikroskopu. V metanolu fiksirana kalcijeva telesca jamske mokrice smo vklopili v sintetično smolo, pripravili ultratanke rezine in na njih izvedli elektronsko difrakcijo (SAED) v presevnem elektronskem mikroskopu. V absolutnem etanolu izolirana kalcijeva telesca *H. riparius* ter sternalne depozite jamske mokrice smo posušili in jih analizirali z mikro-ramansko spektroskopijo.

Ugotovili smo, da je levitveni cikel jamske mokrice daljši kot pri površinskih mokricah, kar sovпада z njenim počasnejšim metabolizmom. Ultrastruktura epitelijskega integumenta med sintezo kutikule je pri jamski mokrici enaka kot pri površinskih mokricah in drugih rakih. Za razliko od drugih mokric pri jamski mokrici po levitvi ne pride do tvorbe dodatnih slojev epikutikule. V levitvenem prostoru so pri jamski mokrici prisotni zunajcelični tubuli, debeli okoli 20 nm, ki se združujejo v snope. Podobni tubuli so bili opisani v levitvenem prostoru tudi pri nekaterih drugih mokricah. V sredini posameznega snopa se nahaja celični izrastek, ki sega do stare kutikule in ga obdaja elektronsko gosta ovojnica. Funkcija celičnih izrastkov ni znana, lahko pa gre za dendrične povezave s čutili v stari kutikuli.

Pri jamski mokrici se v predlevitveni fazi razvijajo sternalni depoziti, ki so sestavljeni iz sferul, manjših od mikrometra. Po zgradbi so podobni sternalnim depozitom pri družini Ligiidae. V primerjavi s preučeni površinskimi mokricami sestavljajo sternalne depozite jamske mokrice manjše sferule kalcijevega karbonata.

Jamska mokrica ima dva para kalcijevih telesc v posteriornih členih pereona. Kalcijeva telesca so epitelijske vrečke, napolnjene z bakterijami in zunajceličnim matriksom, ki vsebuje kisle makromolekule in kristale apatita. Celice epitelijskega kalcijevih telesc imajo nagubano apikalno plazmalemo in vsebujejo veliko mitohondrijev in zrn glikogena. Njihova apikalna površina je obrnjena proti lumnu kalcijevega telesca in je prekrita z zunajcelično ovojnico. V predlevitveni fazi se v kalcijevih telescih oblikuje dodatna

steklasta plast zunajceličnega matriksa, ki je mineraliziran s kalcijevim karbonatom in kalcijevim fosfatom. Ta plast se v polevitveni fazi razgradi. Med resorpcijo kalcija imajo celice epitelijske celice kalcijevih telesc izrazito nagubani tako apikalno kot bazolateralno plazmalemo, medcelični prostori med celicami pa so razširjeni. Kristali apatita in bakterije so prisotni v vseh fazah levitvenega cikla.

Hyloniscus riparius je eden redkih kopenskih enakonožcev, pri katerem se sternalni depoziti v predlevitveni fazi ne pojavijo. V njegovem pereonu se nahajata dva para kalcijevih telesc. Posteriorna kalcijeva telesa so napolnjena z bakterijami, vsebujejo kristale apatita in so podobna kalcijevim telescem jamske mokrice. Anteriorna kalcijeva telesa ne vsebujejo bakterij, zunajcelični matriks v njih pa je mineraliziran s kalcijevim karbonatom. Epitelij kalcijevih telesc je podoben kot pri jamski mokrici, le da v fazi med levitvama apikalna površina celic v anteriornih kalcijevih telescih ni nagubana. V predlevitveni fazi epitelij kalcijevih telesc izloči novo ovojnico. V posteriornih kalcijevih telescih se med staro in novo ovojnico oblikuje steklast sloj matriksa, ki je mineraliziran s kalcijevim fosfatom in kalcijevim karbonatom. V anteriornih kalcijevih telescih se stara ovojnica vgradi v rastoči matriks, mineraliziran s kalcijevim karbonatom. V medlevitveni fazi se najprej začne resorpcija mineralov iz anteriornih kalcijevih telesc. V polevitveni fazi se resorbirajo vse mineralne komponente v anteriornih kalcijevih telescih ter steklast sloj v posteriornih kalcijevih telescih. Med resorpcijo mineralov imajo epitelijske celice v obeh parih kalcijevih telesc močno nagubano apikalno plazmalemo, medcelični prostori med njimi so povečani, v njih pa so prisotne nekaj deset nanometrov velike elektronsko goste granule, ki nastajajo na kratkih izrastkih bazolateralne plazmaleme. Podobne so ultrastrukturne značilnosti transportnih epitelijev med transportom kalcija pri drugih rakih.

Rezultati kažejo, da je prisotnost bakterij v kalcijevih telescih povezana z dinamiko kalcijevega fosfata. Stalna nagubanost apikalne površine epitelijev kalcijevih telesc, ki vsebujejo bakterije, je verjetno povezana z intenzivnim transportom skozi epitelij, ki vzdržuje ustrezne razmere za številčno populacijo bakterij v kalcijevem telescu. Jamska mokrica kopiči v začasnih depozitih kalcija sorazmerno več kalcijevega fosfata kot *H. riparius* in v prostorih med celicami epitelijske celice kalcijevih telesc se pri jamski mokrici med resorpcijo kalcija ne pojavljajo elektronsko goste granule.

Na šestih žleznih organih jamske mokrice se nahajajo raznolike epikutikularne strukture, številne pore, skozi katere izločajo eksokrine žleze ter specializirane senzile. Žleze, ki izločajo skozi šestih žleznih organov, zapolnjujejo večji del pleona. Posamezno žlezo sestavlja nekaj sekrecijskih celic, povezanih z osrednjo celico, in kanalska celica, ki vodi sekrecijske produkte na površino. Osrednja celica je zvezdasta, njeni izrastki segajo globoko v ugreznitve plazmaleme sekrecijskih celic in tako ustvarjajo kanale, ki vodijo sekrecijske produkte proti osrednji celici, preko katere potujejo v kanalsko celico. Takšna zgradba eksokrinih žlez pri rakih še ni bila opisana, v nekaterih značilnostih pa žleze

ščetinastih žleznih organov jamske mokrice spominjajo na krpate žleze mokric. V pleonu in uropodih samcev jamske mokrice se nahajata še dva tipa za samce značilnih eksokrinih žlez, ki so po zgradbi podobne rozetastim žlezam drugih rakov. Ščetinasti žlezni organi najverjetneje izločajo peptide. Žleze samcev jamske mokrice niso podobne predhodno opisanim žlezam samcev rodu *Trichoniscus*, kar nakazuje, da so se v evoluciji razvile neodvisno.

4.2 SUMMARY

Crustaceans possess a calcified exoskeleton (cuticle), which is regularly replaced by molting during growth, reproduction and development. Numerous crustacean groups have evolved various transient calcium deposits, in which calcium is usually accumulated in the form of amorphous calcium carbonate. These deposits most often appear only in specific stages of the molt cycle and likely reduce the dependence of crustaceans on external calcium sources. The synthesis and mineralization of the cuticle and mineralized extracellular matrices of transient calcium deposits are performed by secretory epithelia of the integument and the digestive system.

In terrestrial isopods (Oniscidea), calcium is usually accumulated during the premolt stage of the molt cycle in the form of sternal CaCO_3 deposits. Sternal deposits appear in the ecdysial space (the space between the old and the new cuticle) of anterior sternites of the pereon. The family Trichoniscidae is an understudied group of terrestrial isopods with numerous cave-dwelling representatives. Calcium bodies, special calcium accumulating organs, are present in some trichoniscid woodlice. The molt process and the changes of calcium deposits during the molt cycle have not yet been studied in trichoniscids. Males of some trichoniscids possess gland-piliferous organs, externally visible as porous surfaces covered by cuticular setae. Their structure has thus far been studied in only one species and their function remains unknown.

In our study we monitored the molt of the troglobitic trichoniscid *Titanethes albus* (Koch) in the laboratory and studied the ultrastructure of its integument during the molt cycle. In this species we also studied the structure of the gland-piliferous organs and male-specific exocrine glands in detail. We described the architecture and composition of its calcium bodies and their dynamics during the molt cycle. Our study also included the trichoniscid *Hyloniscus riparius* (Koch), which lives in leaf litter. We also described the structure of calcium bodies and their changes during the molt cycle in this species.

Specimens of *T. albus* and *H. riparius* were maintained in the laboratory. Individuals in different molt cycle stages were fixed in 4 % formaldehyde for the preparation of paraffin sections or fixed in a mixture of paraformaldehyde and glutaraldehyde in cacodylate buffer, postfixed with osmium tetroxide and embedded in synthetic resin for the

preparation of semithin and thin sections. Specimens fixed with the same procedure were dried in hexamethyldisilazane (HMDS), after which samples were attached to holders, sputter-coated with platinum and the external morphology of gland-piliferous organs was studied with scanning electron microscopy.

Histological and ultrastructural characteristics of the calcium bodies during the molt cycle as well as the gland-piliferous organs in intermolt stage specimens were studied with light microscopy. A 3D reconstruction of the spatial distribution of exocrine glands in the male pleon was constructed from serial paraffin sections. The presence of bacteria in tissues was confirmed with *in situ* hybridization of sections with a fluorescent nucleotide probe, specific for eubacterial ribosomal RNA.

Calcium bodies in different molt cycle stages were isolated in methanol and air-dried. Specimens were then attached to holders, fractured to expose the interior, sputter-coated with platinum and observed with a scanning electron microscope. Sternal deposits of *T. albus* were prepared the same way. Several isolated calcium bodies were coated with carbon and their elemental composition was studied with energy dispersive X-ray spectrometry (EDXS) in a scanning electron microscope. Methanol-fixed calcium bodies were also embedded in synthetic resin for the preparation of ultrathin sections. Selected area electron diffraction (SAED) of sections was performed with a transmission electron microscope. Calcium bodies of *H. riparius* and sternal deposits of *T. albus* were isolated in absolute ethanol, air-dried and analyzed with micro-Raman spectroscopy.

We established that the molt cycle of *T. albus* is longer than in epigean woodlice, which corresponds to its slower metabolism. The ultrastructure of the integumental epithelium during cuticular synthesis in *T. albus* is the same as in epigean woodlice and other crustaceans. No postecdysial modifications of the epicuticle, which have been observed in other woodlice, take place in *T. albus*. Extracellular tubules approximately 20 nm in thickness are present in the ecdysial space of *T. albus* specimens in premolt stage and associate into bundles. Similar tubules have been reported in the ecdysial space of other woodlice. At the center of each bundle of tubules, a cellular extension in an electron dense sheath is present and reaches into the old cuticle. The function of cellular extensions is not known, but it is possible that they are dendritic connections with sensory organs in the old cuticle.

During the premolt stage, sternal deposits consisting of spherules under a micrometer in diameter develop in *T. albus*. They are similar in structure to sternal deposits in the family Ligiidae. In comparison with epigean woodlice, the sternal deposits of *T. albus* are composed of smaller calcium carbonate spherules than those of epigean species.

Titanethes albus possesses two pairs of calcium bodies in posterior pereonites. Calcium

bodies are epithelial sacs filled with bacteria and an extracellular matrix, which contains acidic macromolecules and apatite crystals. The apical plasma membrane of cells in the calcium body epithelium is folded and they contain numerous mitochondria and glycogen granules. Their apical surface faces the calcium body lumen and is lined by an extracellular envelope. During the premolt stage, an additional glassy layer of extracellular matrix mineralized with a mixture of calcium carbonate and calcium phosphate is formed in the calcium bodies. This layer is degraded after molt. During calcium resorption, cells of the calcium body epithelium have intensely folded apical and basal plasma membranes and the intercellular spaces between them are dilated. Apatite crystals and bacteria are present within calcium bodies during all stages of the molt cycle.

Hyloniscus riparius is one of the few terrestrial isopods that do not form sternal deposits during the premolt stage. Two pairs of calcium bodies are located in its pereon. The posterior calcium bodies are filled with bacteria, contain apatite crystals and are similar to the calcium bodies of *T. albus*. The anterior calcium bodies, on the other hand, do not contain bacteria and the extracellular matrix within them is mineralized with calcium carbonate. The calcium body epithelium is similar to that of *T. albus*, except that the apical epithelial surface of the anterior calcium bodies is not folded during intermolt stage. During the premolt stage, the calcium body epithelium secretes a new envelope. In the posterior calcium bodies, a glassy layer of matrix mineralized with a mixture of calcium carbonate and calcium phosphate is formed between the old envelope and the new one. In the anterior calcium bodies, the old envelope is incorporated into the growing matrix mineralized with calcium carbonate. During the intramolt stage, mineral resorption starts in anterior calcium bodies first. During the postmolt stage, all mineral components in the anterior calcium bodies and the glassy layer in the posterior calcium bodies are resorbed. The epithelial cells in both pairs of calcium bodies have an intensely folded apical plasma membrane during mineral resorption and the intercellular spaces between them are dilated. Electron dense granules several tens of nanometers in size, which form on short protrusions of the plasma membrane, are present within the intercellular spaces during this time. These ultrastructural features are similar to those reported in other crustacean transporting epithelia.

Our results indicate that the presence of bacteria within calcium bodies is linked to calcium phosphate dynamics. The continuously folded apical epithelial surface in bacteria-filled calcium bodies is likely associated with intensive transepithelial transport, which maintains suitable conditions within the calcium body harboring a large bacterial population. The relative amount of phosphorus accumulated in transient calcium deposits is larger in *T. albus* than in *H. riparius* and electron dense granules are not present in spaces between epithelial cells during calcium resorption from calcium bodies in *T. albus*.

Diverse epicuticular structures, numerous openings of exocrine glands and specialized

senzila are present on the gland-piliferous organs of *T. albus*. Glands which secrete through gland-piliferous organs fill most of the pleon. An individual gland comprises several secretory cells, a central cell, and a canal cell, which leads secretory products to the surface. The central cell is stellar in shape and its extensions reach deep into invaginations of the secretory cell's plasma membranes, thus forming channels leading secretory products towards the central cell, through which they pass into the canal cell. Exocrine glands with similar structure have not previously been described in crustaceans, but several features of the glands in *T. albus* gland-piliferous organs are reminiscent of the lobed glands of woodlice. Two additional types of male-specific glands are present in the pleon and uropods of *T. albus* males. These glands are similar in structure to rosette glands of other crustaceans. Gland-piliferous organs most likely secrete peptides. Male-specific glands in *T. albus* are not similar to previously described male-specific glands of *Trichoniscus*, which indicates that they evolved independently.

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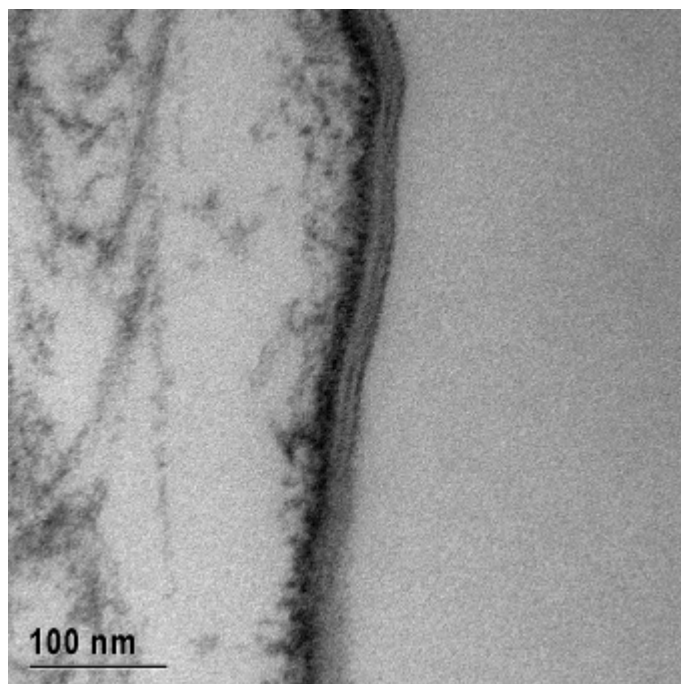
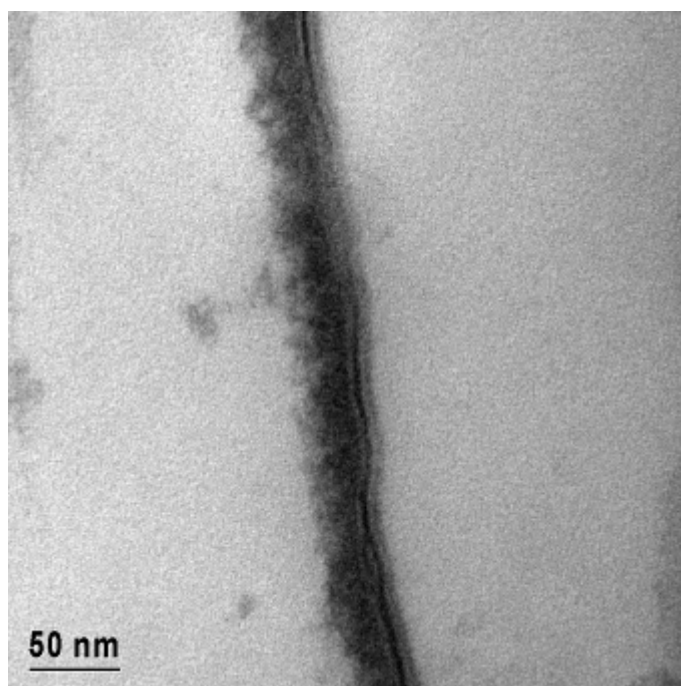
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Alenka Vittori in Aljoša Vodnik sta dali na razpolago stanovanji, v katerih sem lahko spal med doktorskim študijem.

PRILOGE

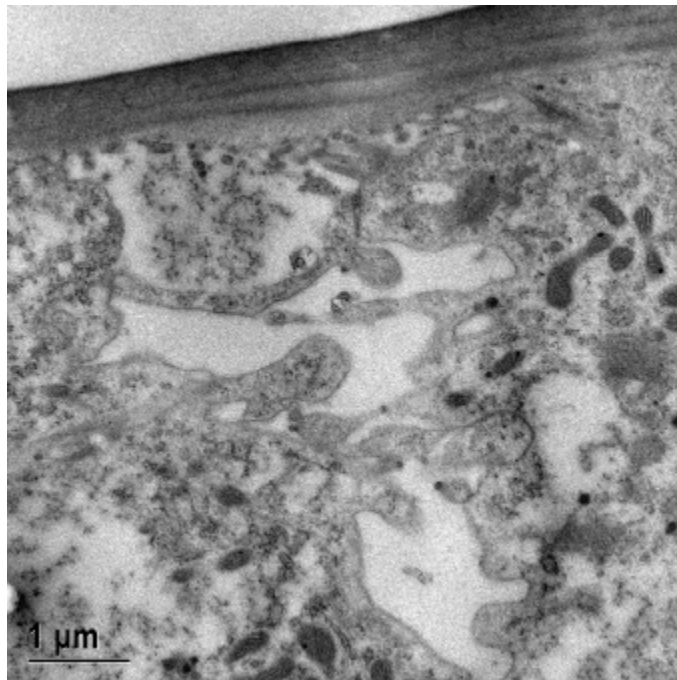
Priloga A

Epikutikula *Hyloniscus riparius* v fazi med levitvama.



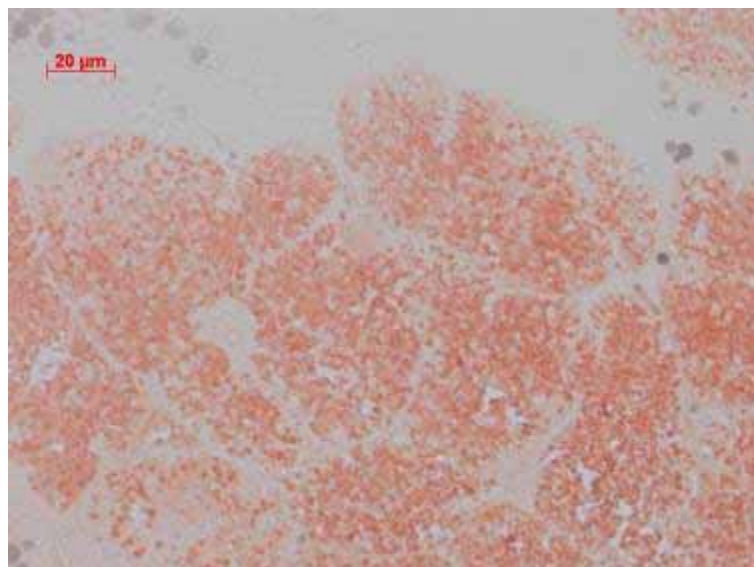
Priloga B

Anteriorni sternalni epitelij jamske mokrice *Titanethes albus* v medlevitveni fazi.

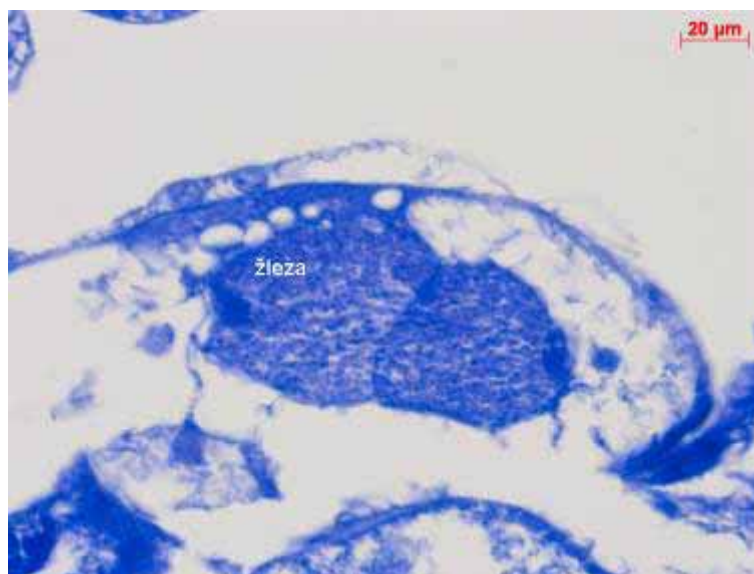


Priloga C

Histokemijska barvanja samčevih žlez tipa 1 jamske mokrice *Titanethes albus*.



Barvanje s Ponceau 2S na poltanki rezini pleona. Proteini se z barvilom obarvajo rdeče. Obarvani so sekrejski vezikli žlez.



Barvanje s Coomassie modrim na parafinski rezini. Proteini se z barvilom obarvajo modro. Sekrejski vezikli žlez so močno obarvani.