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Cutícula e ciclo de muda de duas espécies de isópodos terrestres (Crustacea: Isopoda: Oniscidea)

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“If you're going to be weird, be confident about it”

Sumário

Resumo

Os isópodos terrestres possuem uma cutícula protetora que mantém a forma corporal, permite locomoção e comunicação com o ambiente e protege contra dessecação, infecção e predação. Assim como nos demais crustáceos, a cutícula é composta por uma matriz orgânica que é mineralizada com cálcio. A cutícula é uma estrutura versátil que reflete adaptações ambientais e a ampla distribuição geográfica do grupo. Dessa forma, a ultraestrutura e a composição da cutícula variam entre espécies. Isópodos terrestres fazem mudas frequentes ao longo da vida para crescer e/ou renovar receptores de superfície, o que resulta em reabsorção e deposição cuticular constante. Esse grupo apresenta muda bifásica e deposição de placas de cálcio nos esternitos anteriores antes da ecdise como estratégia para reciclar o cálcio corporal. Estudos relacionados à cutícula nesse grupo contemplam a ultraestrutura, a composição e a deposição bem como o efeito de alguns fatores ambientais na muda. No entanto, poucos estudos exploram as ligações entre ecomorfologia e história de vida. Essa tese visa explorar diversos aspectos relacionados à cutícula e a muda de duas espécies neotropicais de isópodos terrestres. *Atlantoscia floridana* e *Balloniscus glaber* foram usados como modelo uma vez que são frequentemente encontrados nas mesmas localidades e diferem em tipo ecomorfológico e estratégias ambientais. No **Capítulo I**, foram exploradas as estruturas de superfície e a ultraestrutura da cutícula das duas espécies a fim de ver como as diferenças encontradas podem ser relacionadas à história de vida de cada espécie, utilizando técnicas de microscopia. As espécies diferiram em tipo e disposição das estruturas de superfície bem como em espessura e proporção de camadas da cutícula. De maneira geral, as diferenças das estruturas de superfície estão relacionadas à seleção de microhabitat e nicho ecológico. Características cuticulares ajudam a explicar o hábito endógeno de *B. glaber* e epígeo de *A. floridana* enquanto que as diferenças em ultraestrutura são relacionadas a estratégias comportamentais e tolerância ambiental. No **Capítulo II**, o efeito do cálcio alimentar no ciclo de muda das espécies foi testado. Para isso, dieta experimental e análise estrutural da cutícula foram realizadas a fim de entender como diferentes concentrações de cálcio alimentar interferem na duração do ciclo de muda. Peculiaridades na ecdise em *A. floridana* refletem morfologia específica do animal. A duração da intramuda foi maior em *B. glaber* assim como a sobrevivência média nos tratamentos. A duração do ciclo de muda foi influenciada pela concentração de cálcio; uma tendência a menor duração do ciclo com o aumento da concentração de cálcio foi observada em *B. glaber*, enquanto em *A. floridana* a diferença encontrada foi apenas entre o controle sem cálcio e os demais tratamentos. Não houve efeito da dieta no grau de mineralização ou na ultraestrutura em *B. glaber*. Independentemente do tratamento, a maior taxa de mortalidade em laboratório parece estar relacionada com o próprio processo de ecdise, com mortalidade acumulada de 20% do início da ecdise até o início da pós-muda para ambas as espécies. No **Capítulo III**, a secreção da cutícula durante a pré- e pós-muda muda foi observada utilizando microscopia de transmissão. A deposição seguiu o padrão observado para outros isópodos. Entretanto, grânulos eletrondensos presentes no espaço ecdisial durante a pré-muda são provavelmente constituídos de cálcio, sugerindo a reciclagem de cálcio diretamente da cutícula velha para a nova no mesmo segmento. Esses grânulos são depositados nas escamas na nova epicutícula antes da ecdise, sugerindo a presença de cálcio na superfície cuticular das espécies. Além disso, regiões sem a ultraestrutura típica encontrada na pós-muda indicam que há modificação na exocutícula após a ecdise uma vez que a expansão e endurecimento da nova cutícula apenas após a ecdise. De maneira geral, esse trabalho não apenas trouxe novas informações sobre a estrutura cuticular de duas espécies neotropicias, mas também contribuiu para esclarecer conexões entre ecomorfologia e requerimentos biológicos de isópodos terrestres.

Palavras chave: tatuzinho; morfologia funcional; ultraestrutura; exoesqueleto; falha na ecdise; grau de mineralização; cálcio; microscopia

Abstract

Terrestrial isopods have a protective cuticle that maintains body shape, allows locomotion, enables communication with the environment and protects them against desiccation, infection and predation. As in all crustaceans, their cuticle is composed of an organic matrix that is mineralized with calcium. The cuticle is highly versatile reflecting adaptations to environmental conditions and large geographical distribution of this group. Therefore, cuticle ultrastructure and composition vary among species. Terrestrial isopods molt frequently throughout their lives in order to grow and/or renew surface receptors, resulting in constant cuticular resorption and deposition. In this group, this dynamics of cuticle formation is affected by the biphasic molt and by the calcium deposition on sternal deposits prior ecdysis, strategies to recycle body calcium. Studies related to cuticle on this group include ultrastructure, composition and deposition as well as effect of some environmental factors on molting. However, few studies explore connections of ecomorphology and life history of animals. This thesis aimed to explore various aspects of cuticle structure and molting using two Neotropical species of terrestrial isopods. *Atlantoscia floridana* and *Balloniscus glaber* were used as models since they are found in the same locations while differing in ecomorphology and behavioral strategies. In **Chapter I**, I explored the cuticle surface structures and ultrastructure of both species to see how their differences can be related to each species life history traits, using microscopy techniques. Species differed in surface structures type and disposition, as well as cuticle thickness and layer proportion. Overall, differences in surface structure are related to microhabitat selection and ecological niche. Cuticular features further explain the endogeic habit of *B. glaber* and epigeic habit of *A. floridana,* while differences in cuticle ultrastructure relate to behavioral strategies and environmental tolerance. Next, differences on molting cycle and environmental requirements were analyzed. In **Chapter II**, I tested the effects of dietary calcium on the molting cycle of both species. For that, artificial diet and structural analysis of the cuticle were used to understand how different concentrations of dietary calcium interfere with molt cycle duration. Peculiarities were observed during ecdysis in *A. floridana* and reflect to specific morphology of the species. Intramolt duration was longer for *B. glaber* as well as overall survivorship in treatments. Cycle duration was influenced by calcium concentration; a trend of shorter molt cycle length with increasing calcium concentration was observed for *B. glaber,* while in *A. floridana,* only difference between control without calcium and other treatments was observed. Degree of mineralization and cuticle ultrastructure of *B. glaber* showed no difference between treatments. Regardless of treatment or species, higher mortality rate under lab conditions seems to be related to the process of ecdysis itself, with cumulative mortality of 20% from the beginning of ecdysis until the beginning of postmolt. In **Chapter III**, I used transmission electron microscopy to analyze cuticle secretion in both species during pre- and postmolt stages. Cuticle deposition during premolt followed the same pattern as other terrestrial isopods. Nonetheless, electron dense granules present on the ecdysial space during premolt are likely calcium granules, suggesting the recycling of calcium within the same segment. These granules are deposited on the scales of the new epicuticle prior ecdysis, suggesting the presence of calcium on the cuticular surface of both species. Moreover, regions without typical lamellate ultrastructure during postmolt indicate modification of the exocuticle after ecdysis since expansion and hardening must occur after ecdysis. Overall, this work not only added information on cuticular structure of two Neotropical species but also clarified connections between ecomorphology and biological requirements of terrestrial isopods.

Key words: woodlice; functional morphology; ultrastructure; exoskeleton; ecdysial failure; mineralization degree; calcium; microscopy

Apresentação

Esta tese está estruturada em capítulos que correspondem aos artigos desenvolvidos independentemente, mas, que no conjunto, inter-relacionam-se e completam-se. Primeiramente é apresentada uma introdução geral sobre cutícula e muda em isópodos terrestres e os objetivos do trabalho. Todos os capítulos apresentam resultados comparativos entre as espécies *Atlantoscia floridana e Balloniscus glaber* e abordam, respectivamente, a estrutura da cutícula, o processo de ecdise e o efeito de cálcio alimentar no ciclo de muda e as mudanças ultraestruturais da cutícula na pré- e pós-muda. Ao final, são apresentadas as conclusões finais sobre os capítulos bem como perspectivas futuras para trabalhos na área.

Capítulo I – *Morphology, microhabitat selection and life-history traits of two sympatric woodlice (Crustacea: Isopoda: Oniscidea): a comparative analysis*

Neste capítulo são descritas as estruturas da superfície e a ultraestrutura da cutícula de duas espécies simpátricas de isópodos terrestres e discutidas as implicações dessas características ecomorfológicas na história de vida dos animais. As duas espécies apresentam diferenças cuticulares; as estruturas da superfície cuticular ajudam a explicar a seleção do micro-habitat de cada espécie enquanto a ultraestrutura da cutícula tem associação com a estratégia anti-predatória adotada por cada espécie bem como ajuda a explicar a diferença em tolerância a flutuações ambientais previamente observadas para cada espécie.

Capítulo II – *Experimental evidence of dietary calcium influence on molt cycle and cuticular structure on woodlice (Crustacea, Isopoda, Oniscidea)*

Neste capítulo são utilizados experimentos para verificar o efeito do cálcio alimentar no ciclo de muda das duas espécies que possuem diferentes características cuticulares. Os resultados sugerem efeito do cálcio externo no ciclo de muda, sendo esse efeito mais pronunciado na espécie com cutícula mais espessa. Além disso, foi verificada uma alta mortalidade associada ao período correspondente à ecdise e ao início da pós-muda. Essa “falha na ecdise” foi relatada anteriormente em alguns trabalhos, mas dados brutos sobre essa mortalidade são escassos na literatura.

Capítulo III – *Ultrastructural changes in the cuticle of two terrestrial isopod species (Crustacea: Oniscidea) during pre- and postmolt*

Neste capítulo são descritos os processos de reabsorção e deposição da cutícula de duas espécies de isópodos terrestres. O estudo relata não só a sequência de eventos ultraestruturais antes da ecdise, mas também a ultraestrutura da cutícula após a ecdise (pós-muda). A discussão é focada nas estruturas relacionadas à mobilização de elementos constituintes da cutícula velha para a cutícula nova.

.

Introdução geral

Isópodos terrestres

Os isópodos terrestres (Crustacea: Isopoda: Oniscidea) são animais detritívoros de solo que estão envolvidos na ciclagem de nutrientes e na formação do solo. Eles contribuem direta e indiretamente nesses processos e a sua contribuição é afetada por fatores como o tamanho dos indivíduos, da população e a composição de espécies da assembleia (Quadros & Araujo 2008). Esse grupo consiste em aproximadamente 3600 espécies de tamanho pequeno a médio (1,2-30mm) distribuídas por todo o mundo (Schmalfuss 2003). Eles são um dos grupos de macro-decompositores mais abundantes em diversas comunidades (Hassall & Dangerfield 1990, Zimmer 2002a)

Os detritívoros podem apresentar preferência alimentar e diferenciada taxa de consumo de acordo com características das espécies vegetais disponíveis. Essa preferência é relacionada à senescência e ao estágio de decomposição (Wieser 1984, Hassall et al. 1987, Van Wensem et al. 1993, Yeates & Barmuta 1999, Wood et al. 2012), à quantidade de substâncias deterrentes (Cameron & LaPoint 1978, Hassall & Rushton 1984, Targett et al. 1986, Sousa et al. 1998, Canhoto & Graça 1999, Lambdon & Hassall 2005, Wood et al. 2012), à colonização microbiana (Gunnarsson & Tunlid 1986, Kautz et al. 2000, Zimmer et al. 2003, Ihnen & Zimmer 2008) e à disponibilidade de nutrientes importantes (Dudgeon et al. 1990, Graça et al. 2001, Quadros et al. 2014). A colonização microbiana aumenta a atratividade de fontes de alimento para os isópodos, especialmente os de “baixa qualidade” (Ihnen & Zimmer 2008). Quando dada a escolha, os isópodos terrestres consomem folhas de diferentes espécies mantendo uma dieta mista (Wieser 1984) a fim de suprir suas demandas nutricionais, consumindo determinadas folhas pelo seu conteúdo de cobre ou de cálcio, outras para satisfazer suas demandas energéticas enquanto evitam a ingestão excessiva de elementos possivelmente tóxicos (Dudgeon et al. 1990).

Os isópodos terrestres contribuem nos processos de decomposição degradando parcialmente a serapiheira e promovendo a atividade microbiana (Zimmer 2002b). Suas contribuições são indiretas e diretas uma vez que as fezes produzidas são muito mais decompostas que o alimento original. As suas fezes apresentam uma maior superfície de contato e são prontamente colonizados por populações microbianas (Hassall et al. 1987). Essa contribuição indireta para a decomposição da serapilheira por retornar grande quantidade de matéria na forma de fezes (Quadros & Araujo 2008) contribui para a formação do húmus do solo e para o crescimento de florestas (Knoeppay et al. 2000). Tradicionalmente, animais detritívoros eram pensados como possuindo baixa eficiência de assimilação (Szlávecz & Pobozsny 1995). No entando, recentemente, uma revisão de estudos experimentais sobre detritívoros de solo indicou que esses macroartrópodos produzem impactos diretos na mineralização de carbono (David 2014).

Morfologia geral e tipos ecomorfológicos

Os isópodos terrestres são animais segmentados e com um exoesqueleto rígido. Eles apresentam três grupos de segmentos: cefalotórax, pereion (ou tórax) e pleon (ou abdômem). Cada segmento do pereon (sete segmentos) e do pleon (cinco segmentos + pleotelson) consiste em um tergito (placa dorsal) e um esternito (placa ventral) (Sutton 1972) (Fig. 1).

Os isópodos terrestres podem ser classificados em grupos ecomorfológicos (Schmalfuss 1984) de acordo com as suas características morfológicas, as quais são correlacionadas com estratégias ecológicas e padrões comportamentais (Fig. 2). Segundo Schmalfuss (1984) a maioria das espécies de isópodos terrestres pode ser enquadrada em uma das cinco categorias funcionais, exceto os não-conformistas:

- ✓**Corredores** ou “**runners**”: tipicamente possuem corpo estreito, pereópodos longos e tergito liso. Essas espécies correm para evitar predadores.
- ✓**Aderentes** ou **“clingers”:** possuem geralmente tergitos mais largos que os corredores e margem posterior do primeiro epímero côncava (geralmente convexa nas espécies corredoras). Espécies desse grupo geralmente possuem pereópodos mais curtos e apresentam tubérculos ou outros ornamentos na superfície do tergito. Esses animais geralmente permanecem imóveis e aderidos ao substrato quando descobertos.
- ✓**Roladores** ou **“rollers”:** possuem sessão sagital semicircular e capacidade volvacional, com tergitos lisos e se fecham em uma bola para proteger a superfície ventral de predadores.
- ✓**Intersticiais ou “creepers”**: espécies pequenas (comprimento máximo de 5 mm de comprimento) com costelas longitudinais nos tergitos e pereópodos relativamente curtos. Essas espécies habitam buracos no insterstício e estão adaptadas a micro-habitats permanentemente úmidos
- ✓**Espinhosos ou “spiny”**: esse grupo é caracterizado pela presença de espinhos e protuberâncias conspícuas na superfície dorsal. Todas as espécies desse grupo possuem capacidade volvacional.

Além de características morfológicas macroscópicas, características como a espessura e aspecto geral da cutícula que reveste o corpo dos animais também está associada a essas categorias. Espécies com capacidade volvacional como *Armadillidium vulgare* (Latreille, 1804) apresentam, tipicamente, uma cutícula muito espessa e altamente mineralizada. A superfície dorsal é lisa e sem ornamentações, o que dificulta a manipulação e abertura do animal pelo predador. Espécies como *Porcellio scaber* Latreille, 1804 que são aderentes e/ou corredores apresentam uma cutícula mais fina do que as espécies com capacidade volvacional. As espécies corredoras possuem cutícula mais fina, menos mineralizada e mais flexível que espécies de roladores e aderentes (Becker et al. 2005, Hild et al. 2008, Luquet 2012).

Cutícula: estrutura e função

Os artrópodos possuem uma cutícula protetora que sustenta e mantém a forma corporal. A cutícula é uma estrutura extremamente versátil que reflete as adaptações ambientais e a versatilidade ecológica desses animais (Moussian 2013). Além da proteção e da sustentação do corpo, a cutícula possui uma série de funções como proteção contra dessecação e contra predadores de mesma escala, bem como a comunicação com o meio externo (Seidl et al. 2011, Neues et al. 2011, Luquet 2012, Vittori et al. 2012a, Moussian 2013).

A cutícula apresenta diferente ultraestrutura em distintos grupos e partes do corpo. Assim como na maior parte dos crustáceos, a cutícula dos isópodos é composta de quatro camadas: epicutícula, exocutícula, endocutícula e camada membranosa (Travis 1955, Csonka et al. 2013). Abaixo dessa matriz está a epiderme que secreta a cutícula (Fig. 3). As mesmas camadas básicas são encontradas nas cutículas de Hexapoda e de Pancrustacea não-hexapoda; porém, a nomenclatura difere, mas é bem estabelecida para cada grupo (Roer et al. 2015).

A cutícula dos crustáceos consiste em uma porção orgânica composta de fibras de quitina-proteína que são esclerotizadas (i.e. ligação de proteína-proteína e proteína-polissacarídeo) e em uma porção mineral. Na porção orgânica, as fibras de quitina são depositadas em disposição helicoidal sem a presença camadas com deposição preferencial como ocorre em Hexapoda. A porção mineral é constituída principalmente por carbonato de cálcio cristalino (na forma de calcita) e carbonato de cálcio amorfo ("amorphous calcium carbonate - ACC") (Becker et al. 2005). Na cutícula completa e calcificada, a endocutícula é muito mais espessa que a exocutícula. A exocutícula é constituída principalmente por calcita enquanto a endocutícula é composta principalmente de ACC (Hild et al. 2008, Matsko et al. 2011). No total, sete níveis de organização são identificados na cutícula de crustáceos (Fig. 4) que vão desde a polimerização de N-acetil-glucosamina (nível I) até a deposição de carbonato de cálcio ao redor das nanofibras de quitina-proteína (nível VII) (Luquet 2012).

Ecdise e ciclo de muda

Para crescer, os isópodos terrestres realizam mudas periódicas uma vez que o exoesqueleto rígido impede o crescimento contínuo. A duração do ciclo de muda está associada com diversos fatores, como temperatura do ambiente (Steel 1980), estágio de desenvolvimento e período reprodutivo (Lawlor 1976a). Em baixas temperaturas ocorre uma diminuição da frequência da muda ou até uma anecdise temporária (5°-10°C em *Oniscus asellus* Linnaeus, 1758) (Steel 1980).

Os isópodos terrestres apresentam estratégias para reciclagem do cálcio da cutícula uma vez que nesse ambiente a absorção desse elemento não pode ser feita da água como em ambientes marinhos. Uma dessas estratégias é a muda bifásica

(Heeley 1941, Messner 1965, Steel 1982). Os isópodos terrestres realizam primeiramente a muda da região posterior e após, a muda da região anterior, com divisão entre o quarto e o quinto pereonito (Zidar et al. 1998). Esse processo possibilita uma menor perda de $CaCO_3$ (Greenaway 1985, Steel 1993, Ziegler et al. 2007). Além da muda bifásica, os isópodos reabsorvem parte do cálcio cuticular durante a pré-muda e desenvolvem depósitos de cálcio nos esternitos anteriores (Ziegler 1994, 1997, Štrus & Compère 1996, Neues et al. 2011, Vittori et al. 2012a) (Fig. 5).

Quantidades consideráveis de $CaCO_3$ são reabsorvidas da cutícula velha a cada ciclo de muda devido à alta solubilidade do ACC (Becker et al. 2005, Neues et al. 2011). Os íons de carbonato de cálcio e de hidrogênio podem ser rapidamente mobilizados, facilitando a reabsorção destes através do epitélio do integumento. A porção cristalina (calcita) é menos solúvel e é eliminada com a exúvia (Wood & Russell 1987, Becker et al. 2005, Štrus et al. 2008).

Após a perda da exúvia, os animais precisam mineralizar a sua nova cutícula e a mineralização da parte posterior do corpo é acompanhada da reabsorção de mineral da parte anterior do corpo. Isso sugere que os animais utilizam o ACC na mineralização da cutícula para facilitar a reabsorção e a reciclagem do carbonato de cálcio cuticular. A forma amorfa do $CaCO_3$ precede a formação de calcita na camada distal da cutícula do tergito (Hild et al. 2008).

Devido à reabsorção de minerais, a exúvia perdida na intramuda tem menor quantidade de carbonato de cálcio, de material orgânico e espessura que a cutícula da pré-muda inicial (Neues et al. 2011). Ainda assim, parte do cálcio é perdida com a exúvia (Ziegler & Miller 1997). Fontes de cálcio para mineralização da nova cutícula incluem a hemolinfa (Steel 1993), a cutícula da parte posterior do corpo (Becker et al. 2005, Ziegler et al. 2007) e a ingestão da exúvia (Greenaway 1985, Ziegler & Scholz 1997).

Justificativa

Estudos relacionados à muda de isópodos terrestres incluem a frequência da muda (Lawlor 1976b, Steel 1980, Vittori et al. 2012a), a formação e a estrutura dos depósitos e corpos de cálcio (Wood & Russell 1987, Ziegler 1994, Ziegler & Miller 1997, Vittori et al. 2012b, a), a ultraestrutura da cutícula (Štrus & Compère 1996, Ziegler 1997, Vittori et al. 2012a, Vittori & Štrus 2014) e a quantidade e a distribuição de minerais em diferentes espécies e estágios da muda (Becker et al. 2005, Hild et al. 2008, Neues et al. 2011). Algumas conexões foram estabelecidas entre características cuticulares e a biologia das espécies: a composição mineral da cutícula foi relacionada ao grupo ecomorfológico (Becker et al. 2005) e características ultraestruturais e deposição da cutícula com o ambiente de superfície e subterrâneo (Vittori et al. 2017). No entanto, ainda há uma carência de estudos que relacionem características ecomorfológicas com tolerância ambiental, distribuição geográfica e história de vida dos isópodos terrestres (Hornung 2011).

Quanto aos fatores ambientais, pouco se sabe sobre a interferência deles no ciclo de muda dos isópodos, além dos efeitos da temperatura e do fotoperíodo. Espera-se que os animais necessitem de cálcio do meio externo para mineralizar a nova cutícula com mesma espessura e grau de mineralização. No entanto, não se sabe de que maneira a quantidade de cálcio disponível na fonte alimentar afeta a duração do ciclo de muda ou o crescimento dos animais de diferentes tipos ecomorfológicos.

Objetivos

Esse estudo objetivou descrever a ultraestrutura da cutícula na intermuda e durante o processo da muda e compreender como fontes de alimento com diferentes concentrações de cálcio interferem no ciclo de muda e no crescimento de duas espécies simpátricas de isópodos terrestres de diferentes tipos ecomorfológicos (corredor e aderente). Para isso, foram usadas as espécies *Atlantoscia floridana* (Van Name, 1940) e *Balloniscus glaber* Araujo e Zardo, 1995 uma vez que essas são facilmente coletadas e cujas histórias de vida são bastante conhecidas.

Objetivos específicos*:*

1) analisar e descrever a ultraestrutura e estruturas da superfície da cutícula de duas espécies de diferentes grupos ecomorfológicos, utilizando métodos de microscopia óptica e eletrônica;

2) relacionar as características cuticulares com a história de vida das duas espécies;

3) caracterizar e comparar o ciclo de muda e a taxa de crescimento de acordo com as diferentes concentrações de cálcio da fonte alimentar das duas espécies, utilizando experimentos de laboratório com dieta experimental;

4) identificar a composição e o grau de mineralização da cutícula de isópodos submetidos a tratamentos com diferentes concentrações de cálcio alimentar, utilizando análise elementar;

5) descrever e analisar aspectos ultraestruturais relacionados a reabsorção, a deposição e a re-mineralização da cutícula durante diferentes etapas do ciclo de muda.

Espécies modelo:

Para esse estudo, duas espécies de isópodos terrestres foram usadas: *Atlantoscia floridana* e *Balloniscus glaber*. Ambas as espécies são abundantes em diversas localidades do Rio Grande do Sul (Lopes et al. 2005, Quadros & Araujo 2008, Kenne & Araujo 2015), facilitando a coleta para manutenção em laboratório. Diversos aspectos morfológicos e ecológicos como história de vida, dinâmica populacional, curva de crescimento, estratégias reprodutivas e nutrição foram estudadas em ambas a espécies (Araujo et al. 2004a, b, Araujo & Bond-Buckup 2004, 2005, Lopes et al. 2005, Meinhardt et al. 2007, Quadros et al. 2009, 2012, 2014, 2008, Quadros & Araujo 2008, Bugs et al. 2014, Kenne & Araujo 2015).

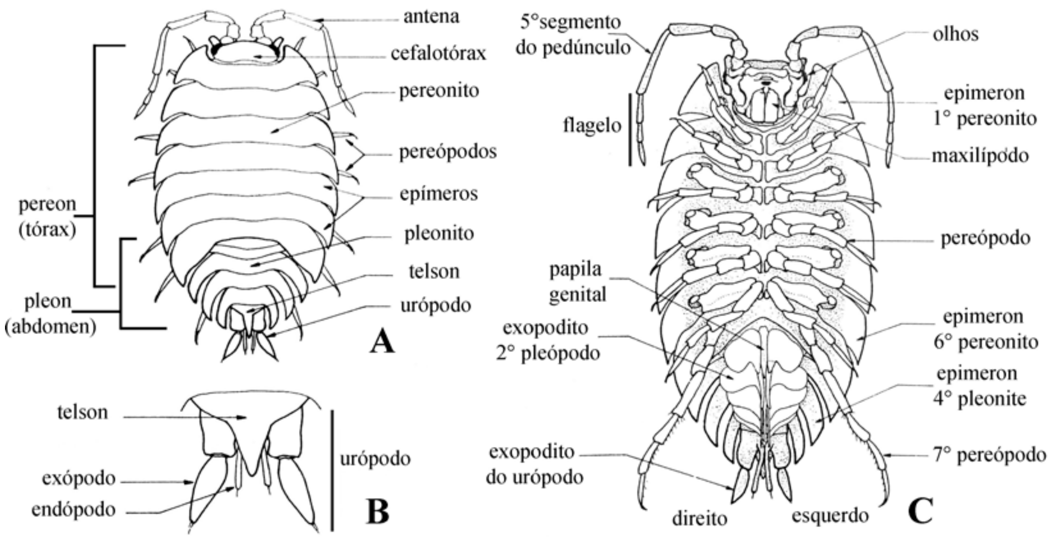

Figura 1. Morfologia geral de um isópodo terrestre mostrando as características externas mais importantes. A) Visão dorsal. B) Detalhe do urópodo. C) Visão ventral. Fonte: Sutton, 1978.

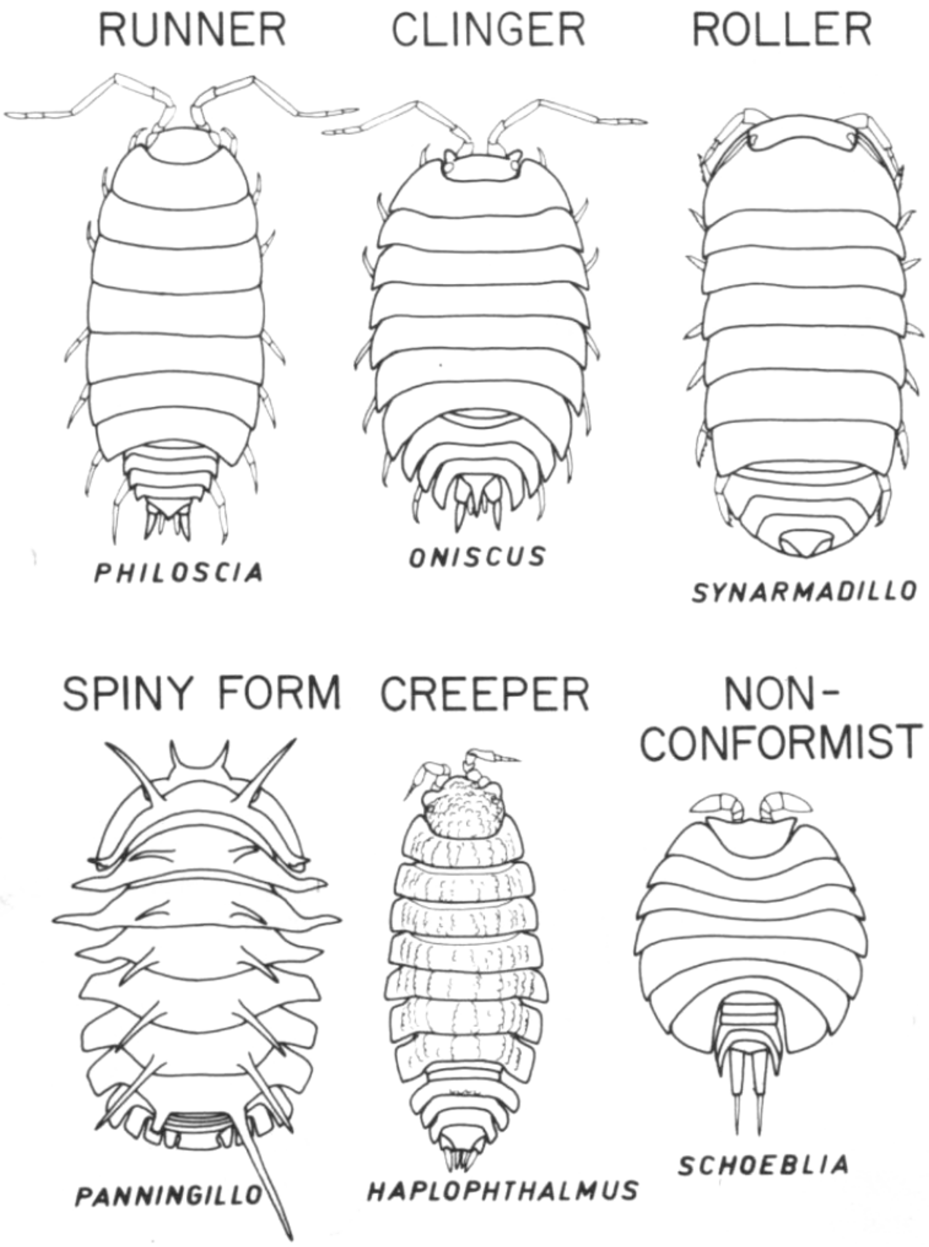

Figura 2. Tipos eco-morfológicos de isópodos terrestres que são relacionados às estratégias anti-predatórias adotadas pelos animais. Fonte: Schmalfuss, 1984.

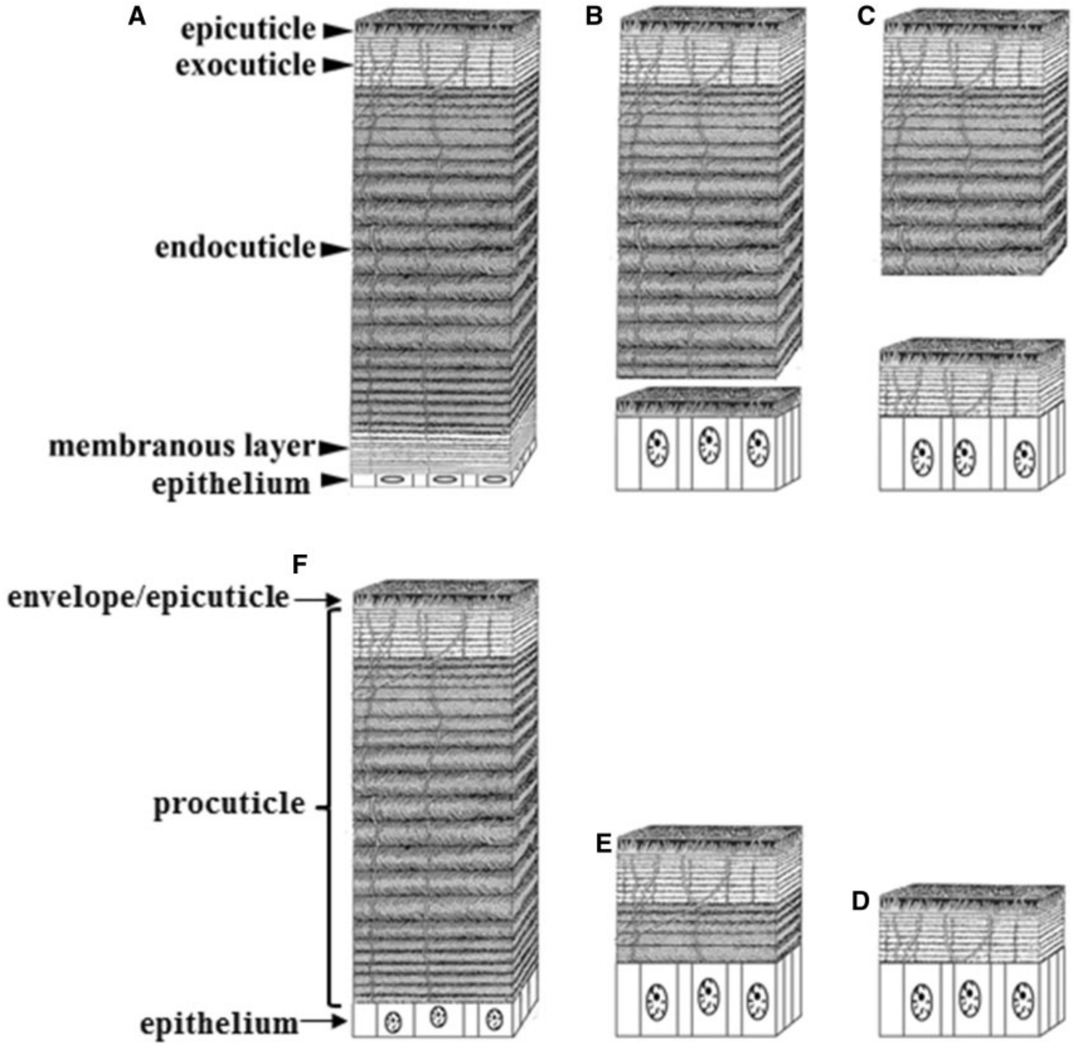

Figura 3. Ultraestrutura da cutícula e epiderme de pancrustacea durante diversas fases do ciclo de muda. A nomenclatura utilizada em pancrustacea está assinalada na figura A e a de hexapoda na figura F. A) Camadas observadas na intermuda. B) O começo da pré-muda é marcado pela apólise (separação da cutícula e epiderme) e seguido do começo da deposição da nova epicutícula e absorção da endocutícula velha. C) Na pré-muda tardia há deposição da nova exocutícula enquanto a enducutícula velha continua sendo absorvida. D-E) Após a ecdise, a nova endocutícula começa a ser secretada pela epiderme e continua até o final da formação da cutícula (F). Fonte: Roer et al, 2015.

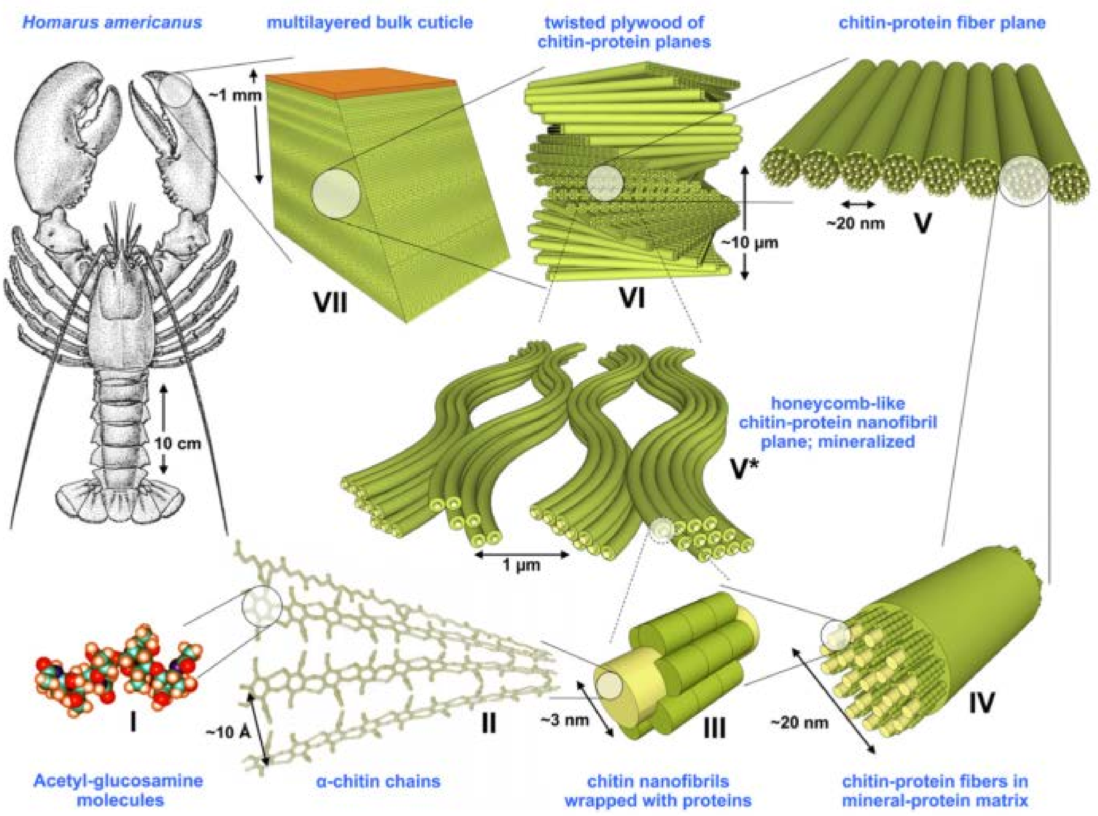

Figura 4. Níveis hierárquicos da organização da cutícula de um crustáceo. Os níveis I a VII representam o modelo geral de formação da matriz da cutícula. Nível I: polimerização de N-acetil-glucosamina. Nível II: formação de cadeias de α-quitina. Nível III: formação de nanofibras de quitina associada a proteínas. Nível IV: formação da matriz mineral-protéica com fibras de quitina-proteína (em *Homarus americanos* esse nível não é observado). Nível V: nanofibras de quitina-proteína em disposição de colmeia; matriz mineralizada. Nível VI: disposição helicoidal de fibras de quitina-proteína. Nível VII: cutícula multilamelada. Fonte: Nikolov et al. 2011.

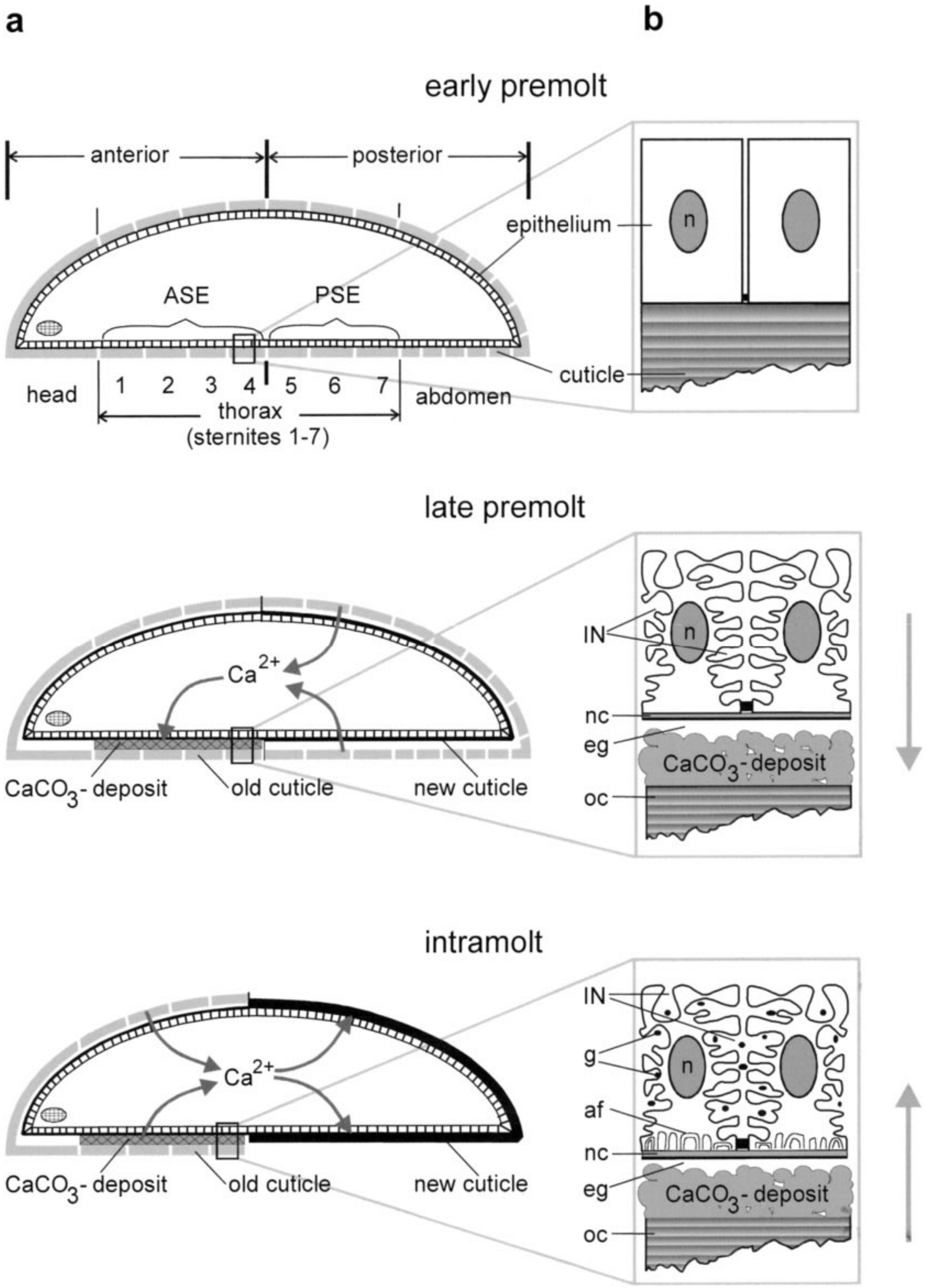

Figura 5. Apresentação esquemática do movimento de íons de cálcio durante o ciclo de muda (a) e da morfologia geral da cutícula (b) do isópodo *Porcellio scaber* em diferentes fazes do ciclo de muda. Fonte: Ziegler & Merz 1999.

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“Science is simply common sense at its best”

Thomas Huxley

Capítulo I

Morphology, microhabitat selection and life-history traits of two sympatric woodlice (Crustacea: Isopoda: Oniscidea): a comparative analysis

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Title Page

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Morphology, microhabitat selection and life-history traits of two sympatric woodlice (Crustacea: Isopoda: Oniscidea): a comparative analysis

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Abstract

The soil is a heterogeneous habitat with layers that differ in many aspects. Soil animals' morphological adaptations relate to specific microhabitat. We compared cuticle surface structures and ultrastructure of *Balloniscus glaber* (endogeic) and *Atlantoscia floridana* (epigeic) using microscopy techniques to understand how morphological characteristics allow sympatry. Species differed in scale and sensillum type and disposition, as well as cuticle thickness and layer proportion. *Balloniscus glaber* has curved scales and robust, caudally oriented sensilla that are most likely adaptive features for endogeic lifestyle. Its cuticle has high overall thickness and numerous stacks that provide toughness compatible with clinging strategy and higher tolerance to seasonal fluctuation. *Atlantoscia floridana* is an epigeic species with flat scales and long perpendicular sensilla, not adapted to the soil layer. Its delicate and flexible cuticle with thinner stacks makes it an agile runner although with lower tolerance to seasonal changes. Overall, differences in surface structure are related to microhabitat selection and ecological niche, so species can occur in same location. Differences in cuticle ultrastructure relate to behavioral strategies and environmental tolerance. This way, knowledge of species' ecology is complemented by morphological data that can help to explain habitat partition.

Keywords

functional morphology; integument; exoskeleton; ultrastructure; scales; tricorn sensilla

1. Introduction

Terrestrial isopods, commonly known as woodlice, are an abundant group of crustaceans that have successfully colonized the terrestrial environment. They feed mainly on leaf litter and decomposing material (Zimmer, 2002). Despite being found from supralittoral to arid zones (Hornung, 2011; Warburg, 1987), they are most commonly found in the litter and soil layers.

The soil is a heterogeneous habitat with layers that differ in many aspects such as organic content, humidity and pore size (Coleman et al., 2004). Some soil animals can migrate into different soil profiles while others are restricted to only one profile (Briones et al., 2009; Krab et al., 2010; Sutton, 1968). Vertical migration is a strategy that enables certain organisms to avoid adverse conditions, such as high temperature and humidity (Fu-xia et al., 2014), provided that the soil presents different microhabitats. A continuum can be observed from epigeic (surface-dwelling species) to endogeic (soil-dwelling) species in many groups including isopods (Sutton et al., 1984). Even though some species of woodlice are considered endogeic, they can still present epigeic activity depending on environmental factors such as time of the day and soil humidity (Tuf and Jerabkova, 2008). Isopods are abundant in many terrestrial ecosystems (Schmalfuss, 2003), but communities of woodlice have typically low species richness and number of dominant species (Hornung et al., 2008; Lopes et al., 2005; Quadros and Araujo, 2008; Vilisics et al., 2012). Although woodlice distribution covers a large geographical range, all species show fairly uniform morphology and little variation in basic body structure.

The whole body is protected by cuticle that functions as an exoskeleton; it supports body shape and provides protection from environmental factors, including predation and desiccation (Seidl et al., 2011; Seidl and Ziegler, 2012). In most crustaceans, this structure is a sclerotized and mineralized matrix secreted by the underlying hypodermis (Compère, 1995). In woodlice, the complex cuticular surface forms various structures such as tubercles, scales, as well as innervated sensilla for communication with the environment (Holdich and Lincoln, 1974). Tricorn sensilla are common exteroreceptors on the dorsal surface and appendages of woodlice (Holdich, 1984) and have mechano- and chemosensory function (Schmalfuss, 1978; Ziegler and Altner, 1995).

The cuticle consists of epicuticle, exocuticle, lamellated endocuticle and membranous layer with several stacks. Some of these layers are periodically reabsorbed while a new cuticle is secreted before ecdysis, resulting in ultrastructural differences throughout the molt cycle. Cuticle architecture varies in different species even though the same layers are always observed in all studied isopods (Hild et al., 2009; Mrak et al., 2015; Neues et al., 2011; Štrus et al., 2008; Vittori et al., 2012; Vittori and Štrus, 2014; Ziegler, 1994) that differs from other arthropods such as insects (Roer et al., 2015).

Cuticle ultrastructure reflects different hierarchical levels of organization that can be related to mechanical properties. For instance, chitin fibers are deposited in helicoidal disposition, forming a plywood structure that resembles lamellae or stacks in transmission micrographs. Fiber arrangement and disposition has been shown experimentally and by modeling to interfere with material toughness (Peterlik et al., 2005). In live animals, mechanical properties are also affected by other aspects as cuticle hydration. Therefore, ultrastructural features indicate when the cuticle is at its most complete form (i.e. intermolt stage) and is a good starting point to understand cuticular mechanical properties in live animals.

Despite having the same overall morphology, woodlice are grouped into several eco-morphological categories (Schmalfuss, 1984) with different behavioral characteristics. Body shape, size of appendices and cuticle characteristics are important characters for this distinction. Rollers such as *Armadillidium vulgare* (Latreille, 1804) can conglobate into a tight ball as a defense mechanism. They typically have smooth tergal surface, making it difficult for the predator to unroll the animal. Clingers such as *Porcellio scaber* Latreille, 1804 either cling to a solid substrate or run to avoid predation. Their cuticle surface often presents tubercles. Species that only run as defense against predators (i.e. runners), usually have lighter, and more flexible cuticle than rollers and clingers although mineralization degree of runners and clinger can be similar (Neues et al., 2007). Cuticle thickness relates to ecomorphological category; however, it is more strongly correlated with microhabitat preference (Csonka et al., 2013).

Woodlice are good experimental models used in laboratory to study several topics ranging from adaptations to land to soil ecotoxicology. While much is known about their morphology, physiology and ecology, other topics as adaptive morphology to environmental conditions and microhabitat distribution still need further investigation (Hornung, 2011). *Balloniscus glaber* Araujo and Zardo, 1995 and *Atlantoscia floridana* (Van Name, 1940) are frequently abundant woodlice species, commonly found in the same locations (Lopes et al., 2005). Nonetheless, these two species present very different characteristics. *Atlantoscia floridana* is a surface-active (epigeic) species that belongs to the runner group, while *B. glaber* is a burrowing soil-active (endogeic) species (Quadros and Araujo, 2007) that can occasionally be found in the litter layer. For these two species, most of the knowledge is restricted to ecology and morphology; ecological studies are mainly related to population dynamics and life history traits (Araujo and Bond-Buckup, 2005; Bugs et al., 2014; Lopes et al., 2005; Quadros et al., 2008; Quadros and Araujo, 2007). Nonetheless, information is somewhat fragmented and few correlations have been made between ecological features and morphological characteristics of the species.

The aim of this study was to compare the cuticle structure and composition of *B. glaber* and *A. floridana*, in order to understand how morphological characteristics relate to microhabitat selection and life history traits. We hypothesize that morphological differences on the cuticle of the selected species are related to epigeic and endogeic nature and to anti-predatory response. For that, we observed the cuticle ultrastructure of the tergites as well as the structures present on its surface using microscopy techniques and related these findings with previous knowledge about ecology of both species.

2. Material and methods

2.1 Sample collection

Specimens were sampled from the leaf litter and topsoil in two locations with co-occurrence of the selected species, near urban areas of Porto Alegre, RS, southern Brazil (30°4'10.19" S, 51°7'10.19" W and 30°20'2.50" S, 51°16'57.82" W). The average cephalothorax width (CW) was 1.9±0.1 mm for *Balloniscus glaber* and 1.1±0.1 mm for *Atlantoscia floridana,* being both species medium sized adults (for growth curves see Araujo and Bond-Buckup, 2004; Meinhardt et al., 2007). After identification, collected specimens were observed for three days and animals with signs of approaching ecdysis (i.e. presence of sternal calcium deposits (Zidar et al., 1998)) were excluded. For each technique, 2 to 5 intermolt specimens of each species were used.

2.2 Sample preparation

Specimens were sacrificed by freezing and fixed in Karnovsky fixative (2.5 % glutaraldehyde, 2 % paraformaldehyde in 0.1 M Hepes buffer, pH 7.3) for at least 3 days at 4 °C. Individuals were then washed in Hepes buffer prior to further steps.

Some tergites were decalcified with EDTA in Hepes buffer solution at 4 °C. Decalcification lasted 4 hours for selected samples of *A. floridana* and 5 days for all *B. glaber* samples. Decalcification times differ due to difference in size and overall toughness. After decalcification, samples were washed in Hepes buffer solution.

2.3 Histology and light microscopy

Paraffin samples were processed using PELCO BioWave microwave tissue processing system. Short times of dehydration by graded concentrations of ethanol were used for *A. floridana* (70 % - 5 min, 95 % - 3 min, 2 x 100 % - 3 min), while slightly longer times were used for *B. glaber* (70 % - 7 min, 95 % - 5 min, 2 x 100 % - 5 min) due to difference in size and toughness of the animal. Infiltration and embedding in paraffin followed.

Blocks were mounted and sectioned parasagittally (8 μm) on rotatory microtome (Leica). Tissue sections were collected on glass slides, deparaffinized with xylene and rehydrated in ethanol series. Slides were stained with Azan (Hubschman, 1962) or Weighert’s hematoxylin-eosin. Sections were photographed with Zeiss Axioskop microscope.

Histological sections were used to measure cuticle thickness. A total of 10 measurements were obtained from the posterior part of the tergite of 3 individuals for each species, using ImageJ software (Abràmoff et al., 2004). As cuticle thickness is correlated to body size (Vittori and Štrus, 2014), average CW of 2.0 and 1.0 mm were estimated from growth curves for *B. glaber* (Meinhardt et al., 2007) and *A. floridana* (Araujo and Bond-Buckup, 2004), respectively, and therefore used to normalize cuticular measurements. To make comparison easier, figures of animals were oriented with the head to the left-hand side.

2.4 Transmission electron microscopy (TEM)

For ultrastructural analysis, some of the samples were post-fixed in osmium tetroxide for one hour and washed in buffer solution. Samples were dehydrated in ethanol series (30%, 50%, 70% and absolute for 20 min each). The final ethanol solution was gradually replaced by acetone and specimens were embedded in Agar 100 resin. Polymerization of the resin was performed in embedding molds at 60 °C for 48 h.

Parasagittal semithin and ultrathin sections were cut with Reichert Ultracut S ultramicrotome (Leica). Semithin sections (0.5 μm) were cut with a glass knife and stained with Azur II – Methylene Blue. Images were obtained with a Zeiss AxioImager Z.1 light microscope, equipped with an HRC Axiocam camera and Axiovision software. Ultrathin sections (70 nm) were floated on water, collected on copper grids, and contrasted with 4% uranyl acetate and 10 % lead citrate. Grids were examined with a CM 100 transmission electron microscope (FEI). Micrographs were recorded with BioScan 729 and Orius 200 cameras (Gatan) using Digital Micrograph software (Gatan). On the micrographs, animals are oriented with the head to the left-hand side.

2.5 Scanning electron microscopy (SEM)

Fixed samples were washed with 0.1 M Hepes buffer, dehydrated in an ascending ethanol series and transferred to pure ethanol. After gradual substitution of ethanol with hexamethyldisilazane (HMDS), samples were air-dried and attached to metal holders with conductive silver paint. Mounted specimens were sputter-coated with platinum and observed with a JSM-7500F field emission scanning electron microscope (JEOL).

3. Results

The cuticle of both species exhibit comparable general ultrastructure but differ considerably in thickness and sculpturing. The layers differ in thickness and proportion in both species as well as fiber arrangement. The epicuticular surface of the tergites of both species comprises scales and sensilla and lacks tubercles and other ornaments. Shape, size and distribution of the scales and sensilla differ between species and can help to explain habitat partition.

3.1 *Balloniscus glaber*

3.1.1 Scales

The cuticular surface of the tergites of *Balloniscus glaber* (Fig. 1) presents fine scales and sensilla scattered throughout the dorsal surface (Fig. 2B). The base of the scales is rounded and the distal end curves anteriorly (Fig. 2C). In some areas they overlap. The profiled surface of the scales extends in longitudinal direction and forms a serrated edge at the curved distal end of the scale (Fig. 2D-F). Although scales cover the entire tergite, they appear more scarce and shorter on the part covered by the preceding tergite. Areas with removed scales are present on both, covered (anterior) and exposed (posterior) areas of the tergites (Supplementary Material).

3.1.2 Sensilla

Scales are always observed in front of each sensillum (Fig. 2A). Although not as densely distributed as scales, the sensilla are found throughout the entire tergite

(Fig. 2B). These exterior receptors comprise a sensory hair with an apical pore, embedded in a shaft-forming cuticular structure at its base, without observable articulation with the basic cuticle (Fig. 2). The shaft opens dorsally towards its distal end, exposing the sensory hair of the sensilla. Regardless the sensillum morphotype, the distal ventral end always exceeds the length of its sensory hair (Fig. 2B, D-F).

The sensilla are oriented caudally, protruding gently from the cuticle in an acute angle with the body surface (Fig. 2). Besides their scattered distribution throughout the surface of the tergites, a line of sensilla is also observed at the edge of the posterior margin of all tergites (Fig. 2B, C). Although the surface-type and the edge-type sensilla have the same basic anatomy, they differ in size and shape.

The edge-type sensillum (Fig. 2A, C) is generally longer than the surface-type, measuring about 30 µm. The protective shaft of the edge-type sensillum resembles a cluster of filaments merged at their base and slightly fanning towards their distal end (Fig. 2A). Although the overall structure of this sensillum type is about the same throughout the entire edge of the tergite, the length of the sensory hair and supporting ventral side varies without any clear pattern and regardless of the position of the sensillum, so the apical pore can be located proximally or distally in the sensillum (Fig. 2C).

The surface-type sensillum (Fig. 2D-F) does not change much in shape, but varies considerably in size. It measures from 15 to 25 µm. In this sensillum type, the protective shaft consists of merged filaments rather than a cluster of filaments. Regardless of the size of the sensillum, the sensory hair always extends distally, towards the tip of the supporting ventral side.

3.1.3 Cuticle ultrastructure

Histological sections show tergites arranged as overlapping series of successive segments (Fig. 1). In these sections, no scales were observed in the anterior part of the tergite. The overlapped scales consist of epicuticle only (Fig. 3E). In transmission electron micrographs, a thin epicuticle composed of two electron dense layers is observed (Fig.3C). This layer forms scales (Fig. 3E) on the cuticle surface. These are thicker at their base (approximately 400 nm) and thinner distally. The tergal cuticle is composed of distinct layers: epicuticle, distal and proximal exocuticle, and endocuticle with membranous layer facing the epithelium (Fig. 3 A, B). The protective shaft from the sensillum (Fig. 3F) is also constituted of epicuticle material.

As the cuticle thickness is slightly different in the anterior and posterior parts of the tergite, measurements were obtained from the posterior part. The average thickness of the cuticle is about 21±1 µm, although average thickness of one individual was around 30 µm. The epicuticle (Fig 3C) consists of several layers (Fig. 3C) with overall thickness about 100 nm. The exocuticle (Fig. 3A-C) measures 4-5 µm on average and presents several stacks. It is differentiated in distal and proximal layers that are most noticeable at the posterior edge of the tergite (Fig. 3B). The endocuticle is the thickest layer comprising numerous stacks or lamellae (Fig. 3A). In this layer, the distal stacks are thicker than the proximal stacks. The average thickness of the postecdysial layers (endocuticle and membranous layer) is 18±1 µm with average stack thickness of 0.7±0.07 µm. For the individual with thicker cuticle, the average exocuticle thickness did not differ from other animals, but average endocuticle thickness was around 23 µm. Pore canals (Fig. 3D) are observed in all length of the cuticle but are more abundant in the anterior part of the tergite.

3.2 *Atlantoscia floridana*

3.2.1 Scales

The tergites of *A. floridana* (Fig. 4A) present a smoother surface than *B. glaber* (Supplementary Material). The cuticular scales are flat, close to the surface, semi-circular at the base and rounded at their distal end (Fig. 5A). They rarely overlap. The profiled surface of the scales extends longitudinally and gradually wrinkles towards the distal end of the scale, forming an irregular yet smooth distal edge. The posterior edge of the tergites ends with wide, rectangular, flap-like scales, extending alongside the tergite edge (Fig. 4B, 5D).

3.2.2 Sensilla

The base of the sensillum protruding from the tergites of *A. floridana* is always overlapped by a scale (Fig. 5B, C). When preceding the sensillum, the scale usually projects upwards rather than lying flat along the surface (Fig. 5B). The sensilla on the surface of *A. floridana* also comprise a sensory hair with an apical pore and a supporting cuticular shaft around the sensory hair (Fig. 4C, 5B-C). Two kinds of sensilla were observed on the surface of *A. floridana*: long-type (Fig. 4C, 5B) and short-type sensillum (Fig. 5C, 6C). Both types are scattered throughout the tergite surface and absent at the posterior edge of the tergite in this species (Fig. 6D).

The long-type sensillum (Fig. 6B) measures about 40 µm and protrudes perpendicularly from the surface. It consists of a long, tube-like shaft forming a short, shallow gutter supporting the posterior face of the sensory hair, which extends beyond the surrounding shaft. This structure cuts through the cuticle ending in a bulb with nervous tissue (stained in red with Azan) underneath the cuticle (4C). The apical pore at the tip of the hair is anteriorly oriented (Fig. 5B).

The short-type sensillum (Fig. 5C, 6C) is much shorter than the previous type, measuring about 10 µm. They point backwards and lay flat on the surface. The flat, leaf-like shaft opens dorsally, exposing the sensory hair, which never extends beyond the edge of the supporting ventral side.

3.2.3 Cuticle ultrastructure

Histological sections show the overlapping tergites of the successive segments, with the flap-like scale at the edge forming an awning structure towards the next segment (Fig. 4A-B). In histological sections, scales are not easily observed provided that they are flat to the surface. Both types of scales consist of epicuticle. The scales are thinner comparing to those of *B. glaber* (approximately 200 nm), and have constant thickness throughout their length. The protective shaft from the sensilla is also constituted of epicuticle material (not shown). In transmission micrographs, the cuticle structure shows three distinct layers, a thin epicuticular layer, an exocuticle, and an endocuticle with pore canals (Fig. 6).

The cuticle of *A. floridana* (Fig. 6A) is about 6±0.3 µm thick in average. Maximum thickness recorded was around 8 µm. The exocuticle is about 1.5±0.1 µm thick and differentiation of proximal and distal parts that can be observed most clearly at the posterior margin of the tergite (Fig. 6B). Although thinner than in *B. glaber*, the relative proportion of the exocuticle is higher in *A. floridana* (Fig. 7). The endocuticle is about 4-5 µm thick. The average stack thickness from the postecdysial layers (endocuticle and membranous layer) is 0.3±0.02 µm, with thinner stacks on the proximal end.

4. Discussion

This study presents new information on cuticular morphology of two woodlice species and correlates this ecomorphological data with microhabitat selection and life history traits. We show the differences in cuticular surface structures, and ultrastructure of two woodlice species with similar ecological requirements that allow them to occupy different microhabitats within the soil.

4.1 Surface structures of Balloniscus glaber *and endogeic lifestyle*

Balloniscus glaber has cuticular surface structures that allow burrowing behavior and endogeic (soil-active) lifestyle, despite the lack of some morphological attributes previously related to this lifestyle. In fact, animals were more frequently collected from the soil than from the litter in both selected locations (Wood, C.T., personal observation). Previously, endogeic species have been mainly related to the creeper ecomorphological group that inhabits crevices in the soil (Sutton 1984). Unlike creepers that are small and narrow species (Sutton et al., 1984), *B. glaber* is larger and does not have ornamentations such as tubercles. Instead, they present many curved scales that protrude upwards. These structures are considered to be anti-adhesive (Schmalfuss, 1976) so their high density throughout the dorsal surface can help preventing attachment of small soil particles, despite the lack of tubercles and ribs observed for this species.

Another characteristic that is compatible with endogeic activity and burrowing behavior is the position of sensilla. They present an acute angle with the body surface which means that the receptors are less prone to breaking than perpendicular sensilla. Also, the caudal orientation makes the apical pore less prone to blockage by soil particles. The same disposition has been shown on the clinger species *Porcellio scaber* (Ziegler and Altner, 1995). Furthermore, the protective structure of the sensilla around the sensory hair is quite robust and should be resistant to breaking in a compact environment like the soil. Therefore, the observed disposition and overall external morphology seem to be related to the surrounding physical environment and endogeic nature of *B. glaber*.

4.2 Cuticle ultrastructure of Balloniscus glaber *and clinging strategy*

Although *B. glaber* possess an intermediate morphology between runners and clingers, the observed cuticle ultrastructure is similar to that of other clinger species. Clingers require a tougher cuticle than runners since they attach firmly to the substrate to stop the predator from accessing their soft parts. We observed a similar cuticular thickness between *B. glaber* and *Porcellio scaber* (Hild et al., 2008) that also present clinging strategy.

Besides thickness, specific cuticle ultrastructure is also related to mechanical strength in arthropods. Mechanical properties have been related to fiber arrangement and hierarchical level of organization (Erko et al., 2013) and organic and mineral composition (Bar-On et al., 2014; Erko et al., 2015, 2013; Huber et al., 2014; Nikolov et al., 2011; Politi et al., 2012; Wu et al., 2016). For example, great changes in fiber orientation along the cuticle are suggested to influence mechanical properties and its resistance to shear in subterraneous woodlice (Vittori and Štrus, 2014). Therefore, a heterogeneous cuticle with a thick proximal exocuticle layer and an endocuticle with a high number of stacks of varying thickness suggests that the cuticle of this species possesses high mechanical strength in live animals.

In terms of behavior, *B. glaber* can present tonic immobility when disturbed (Quadros et al., 2012) and clings tightly to the substrate when attacked. Morphologically, it also possesses some attributes common to clinger species (Schmalfuss, 1984) such as short pereopods and flat body. This indicates *B. glaber* is a clinger and cuticle thickness and ultrastructure might be an additional characteristic related to anti-predatory response.

The presence of five pairs of pleopodal lungs (Araujo and Leistikow, 1999) along with the higher cuticle thickness of *B. glaber* can be related to the higher environmental tolerance observed for this species. Morphological characteristics such as surface area of respiratory structures and total cuticle thickness influence animal's environmental tolerance and therefore are important factors influencing habitat distribution (Csonka et al., 2013). This species is resistant to seasonal fluctuations regarding temperature and humidity (Quadros and Araujo, 2007), so the thickness of the cuticle may also be related to this observed tolerance. Furthermore, the ultrastructure of the epicuticle with two electron dense layers can also be related to more effective waterproofing.

4.3 Surface structures of Atlantoscia floridana *and epigeic lifestyle*

Unlike *B. glaber*, the cuticular surface of *A. floridana* doesn't present characteristics compatible with endogeic (soil active) habit. This species is characteristic of the litter layer with few individuals occurring in the most superficial soil layer during cold periods (Araujo and Bond-Buckup, 2005). *Atlantoscia floridana* has small body size and lacks surface structures that could minimize water adhesion on the body surface. Also, many perpendicular sensilla observed in *A. floridana* were broken on scanning micrographs even though the preceding flat scale at the base of the sensilla might help in stopping it from moving frontwards. These surface structures may restrict *A. floridana* to the surface layer (i.e. litter).

Exposition to the environment and predators on the surface layer may also provide sufficient evolutionary pressure for the development of the observed disposition of sensilla in this species, which may increase perception of the surrounding environment. The litter layer can be used as predation arena for soil animals (Kajak, 1995), and detection of substrate vibration can be a trigger for anti-predator response (Lohrey et al., 2009). Therefore, subtle movements in the litter may elicit a running response in this species so the long-type sensillum may increase the potential surrounding area which the animal can perceive. Both sensilla types present a bulb with nervous tissue underneath the cuticle, but more information on their ultrastructure is needed to determine chemosensory function.

4.4 Cuticle ultrastructure of Atlantoscia floridana *and running strategy*

The cuticle ultrastructure observed in *A. floridana* indicates a delicate and flexible nature that is compatible with running strategy. The cuticle thickness of this species is similar to other surface trichoniscids (Vittori and Štrus, 2014), and the ultrastructure similar to other runners such as *Ligia italica* Fabricius 1798 (Štrus et al., 2008) and *Titanethes albus* (C. Koch, 1841) (Hild et al., 2009; Vittori and Štrus, 2014). Besides the thickness, the more homogeneous and looser fibrilar structure of the cuticle of *A. floridana* also indicates its light nature. Further testing on mechanical properties should be conducted to confirm these features.

The thin and flexible cuticle of *A. floridana* is compatible to its running strategy but might be less effective in preventing accumulation of unwanted particles between segments. Also, the overlapping area of the tergites in this species is narrower than in *B. glaber* since the length of this area is related to overall body size. Thus, the flap-like scale at the posterior edge of the tergites constitutes an additional physical barrier that protects the arthrodial membrane, and might increase protection against entrance of unwanted soil particles in the space between tergites. This flap-like scale might work in a similar way to the wedge-shape and the elongated scales with long bristles found in *Tylos europaeus* Arcangeli, 1938 (Seidl et al., 2011).

Populations of *A. floridana* are more unstable than of *B. glaber* (Quadros and Araujo, 2007) and species' abundance is highly affected by fluctuations in temperature and humidity (Araujo and Bond-Buckup, 2005). The comparably thinner cuticle observed in this species might make it less tolerant to environmental changes. Other physiological attributes related to environmental tolerance are not known for this species and could also interfere in population stability. Moreover, the observed surface structures seem to restrict species to the surface-layer and hinders vertical migration (not recorded for this species in the literature) that would help the animal to avoid certain adverse environmental conditions.

5. Conclusions

Overall, we observed that surface structures present in *Balloniscus glaber* such as scale type and disposition of sensilla are adequate for endogeic lifestyle, while the cuticle ultrastructure with attributes such as higher overall thickness and stack number indicates this species has a tough cuticle that is necessary for clinging anti-predatory response. On the other hand, the size and disposition of sensilla and flat scales found on the surface of *Atlantoscia floridana* coupled with its thinner and delicate cuticle makes it an agile epigeic runner. Since cuticular characteristics affect physiological attributes such as resistance to desiccation, these have implications on resistance to environmental fluctuations. Therefore, population stability of both species can be not only related to animal’s behavioral and reproductive strategies but also to differences in physiology influenced by cuticular characteristics.

This work described morphological attributes from the cuticle of two species and combined knowledge on ecology to help to understand how they relate to habitat partition and life history. Functional morphology data can help to establish connections between morphology and ecology and provide future directions and experiments to further elucidate the inferred connections.

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Appendix - List of figures

Fig. 1. Parasagital histological sections of *Balloniscus glaber* stained with Azan showing overlapping tergites.

Fig. 2. Scanning micrographs of sensilla with preceding scale and their distribution on the tergite cuticle of *Balloniscus glaber*.

Fig. 3. Transmission electron micrographs of the cuticle structure of *Balloniscus glaber*.

Fig. 4. Histological sections of *Atlantoscia floridana*.

Fig. 5. Scanning electron micrographs of sensilla with preceding scale on the cuticular surface of *Atlantoscia floridana*.

Fig. 6. Transmission electron micrographs of the cuticle structure of *Atlantoscia floridana*.

Fig. 7. Relative thickness of cuticular layers and transmission electron micrographs of the cuticle ultrastructure of *Balloniscus glaber* and *Atlantoscia floridana*.

Supplementary material:

Fig. Dorsal cuticular surface of *Balloniscus glaber* (A-C) and *Atlantoscia floridana* (D-F).

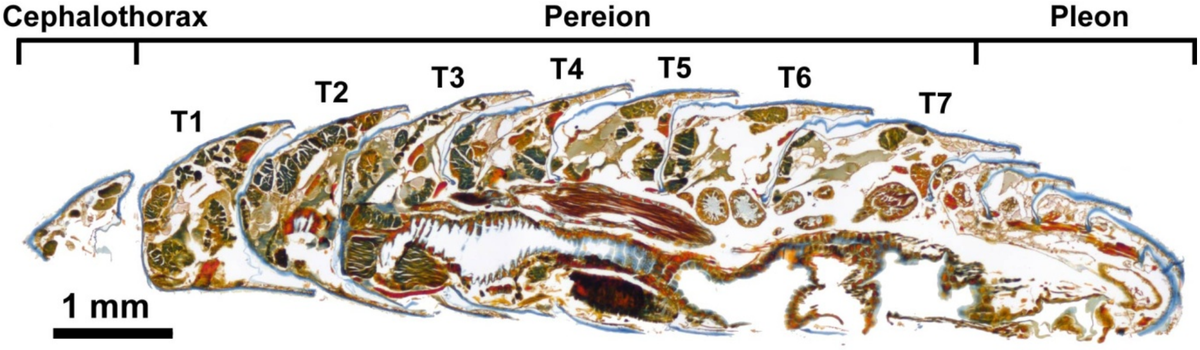


Fig. 1. Parasagittal histological sections of *Balloniscus glaber* stained with Azan showing overlapping tergites.

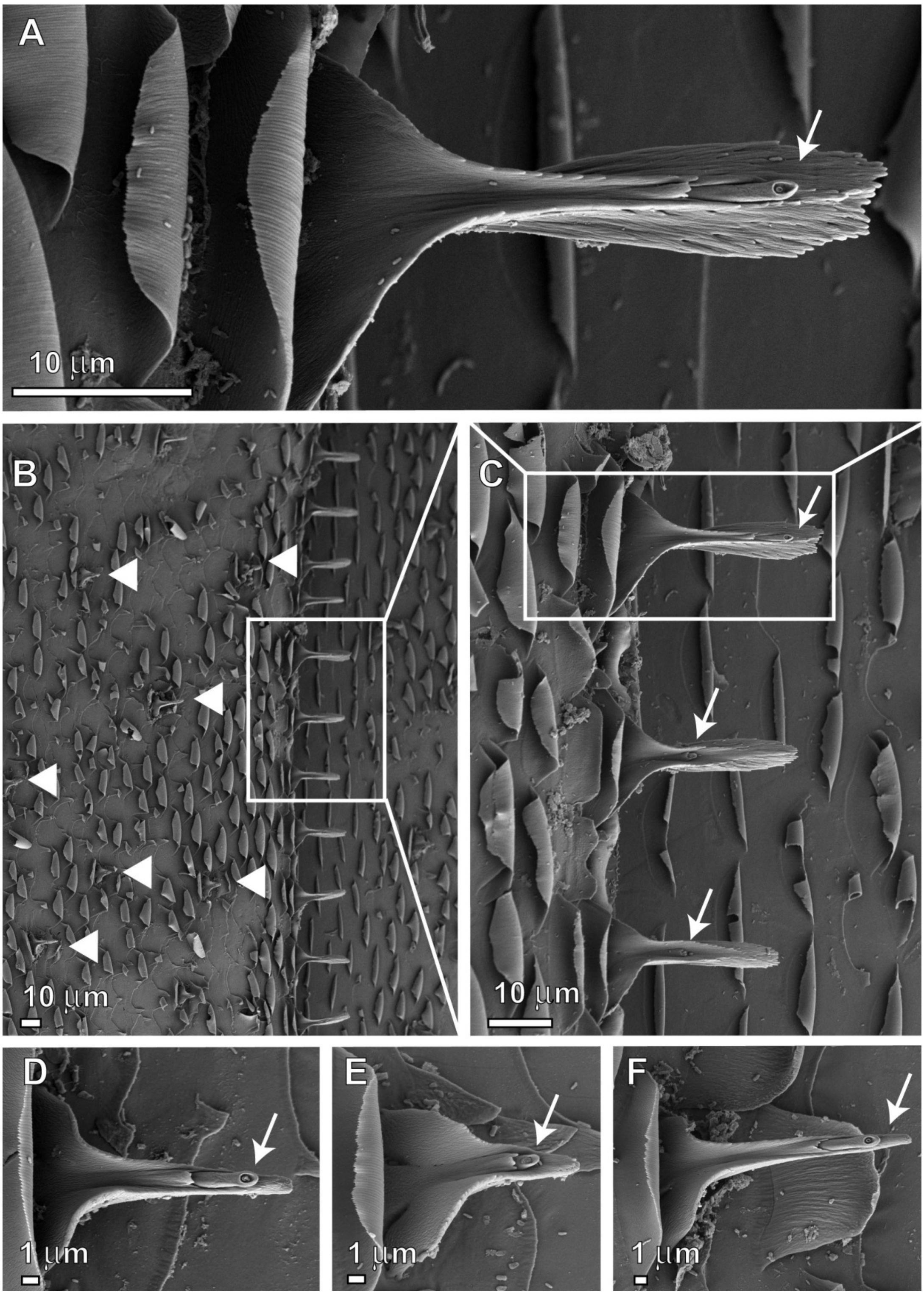


Fig. 2. Scanning micrographs of sensilla with preceding scale and their distribution on the tergite cuticle of *Balloniscus glaber*. A) Detail of edge-type sensillum comprising a sensory hair with an apical pore (arrow) surrounded by a tapering tubular projection. B) Tergite surface showing scattered scales, surface-type sensilla (arrowheads) and a line of edge-type sensilla. C) Edge-type sensilla with distally and proximally positioned apical pores (arrows). D-F) Several morphotypes of surface type-sensilla (arrows indicate apical pores). Note the serrated margin of the curved scale visible on figures D-F.

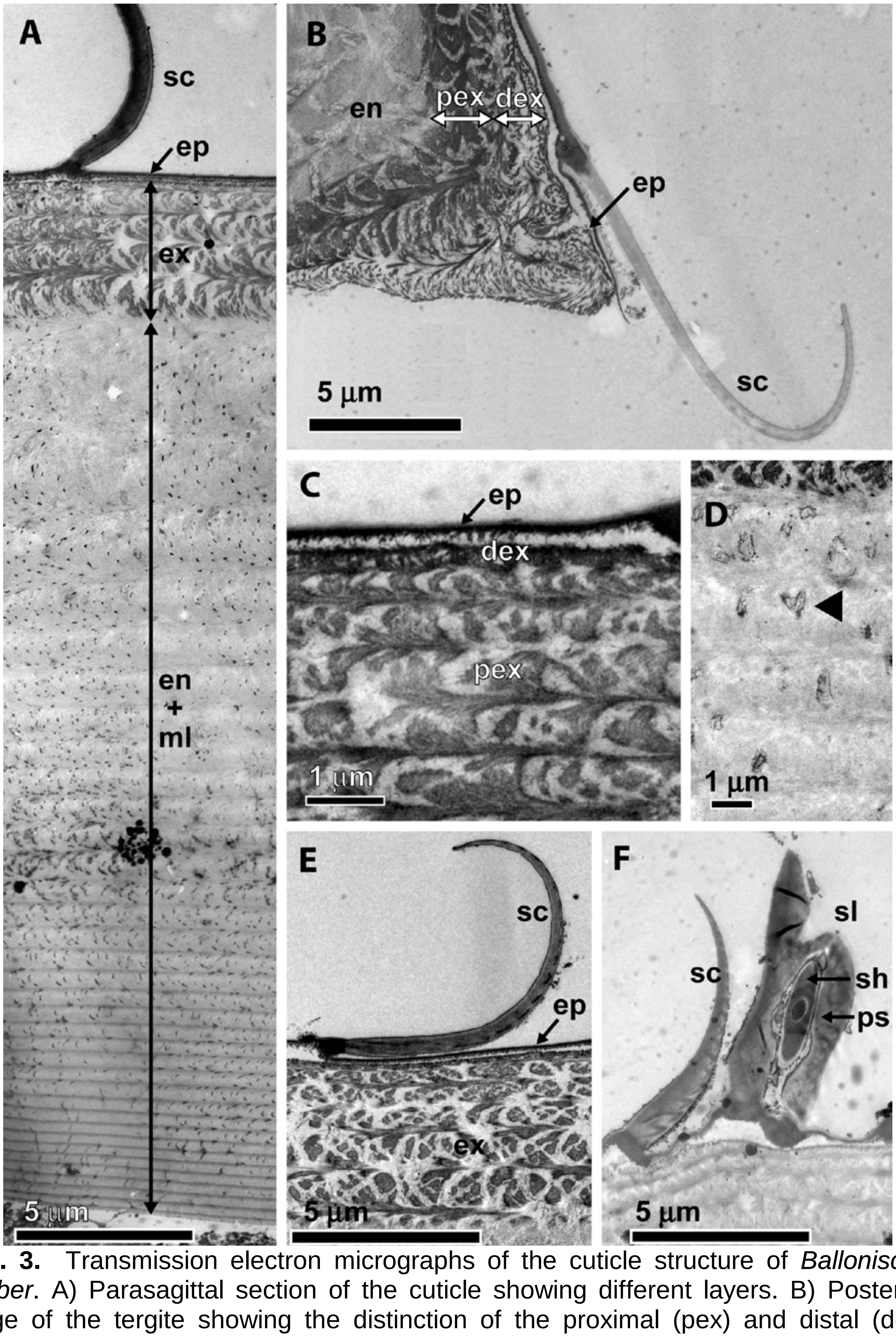


Fig. 3. Transmission electron micrographs of the cuticle structure of *Balloniscus glaber*. A) Parasagittal section of the cuticle showing different layers. B) Posterior edge of the tergite showing the distinction of the proximal (pex) and distal (dex) exocuticle. C) Detail of epicuticle and exocuticle. D) Transversal cut of pore canals (arrowhead) in the endocuticle. E) Curved epicuticular scale on the tergite surface F) Surface-type sensillum with overlapping scale. sc, scale; ep, epicuticle; ex, exocuticle; en, endocuticle; dex, distal exocuticle; pex, proximal exocuticle; ml, membranous layer; sl, sensillum; sh, sensory hair; ps, protective shaft.

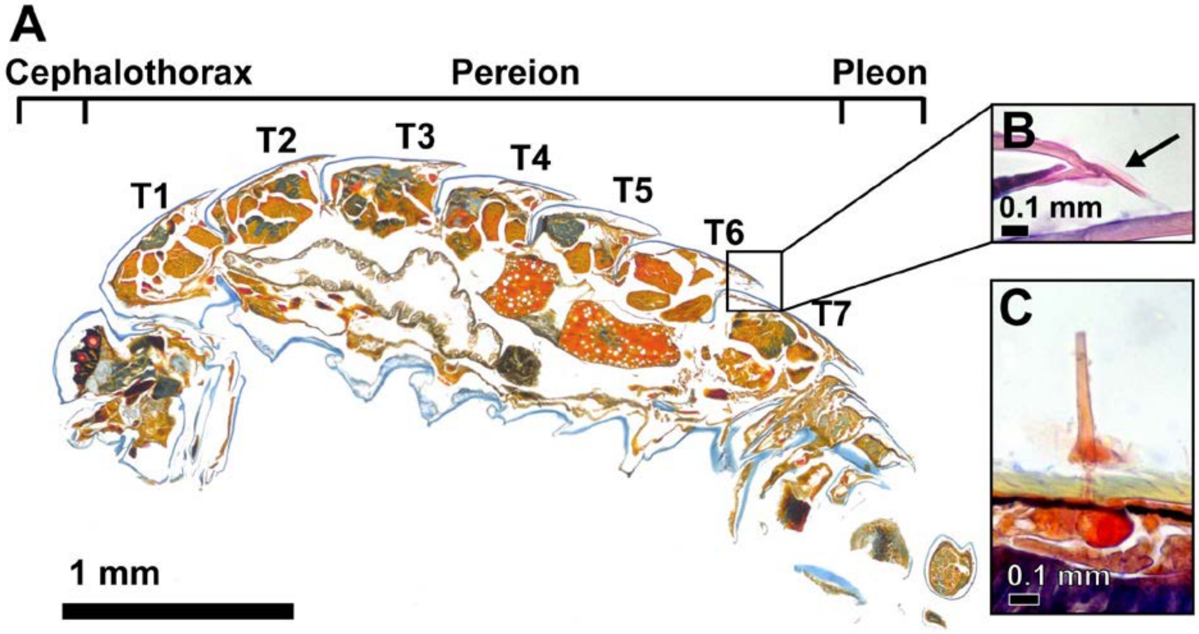


Fig. 4. Histological sections of *Atlantoscia floridana*. A) Parasagittal section stained with Azan showing overlapping tergites (premolt animal). B) Hematoxylin-eosin staining of flap-like scale (arrow) at the posterior edge of the tergite on the area equivalent to the one assign on figure A. C) Azan stained transverse section of long-type sensillum with bulb containing nervous tissue (red-stained) underneath the cuticle.

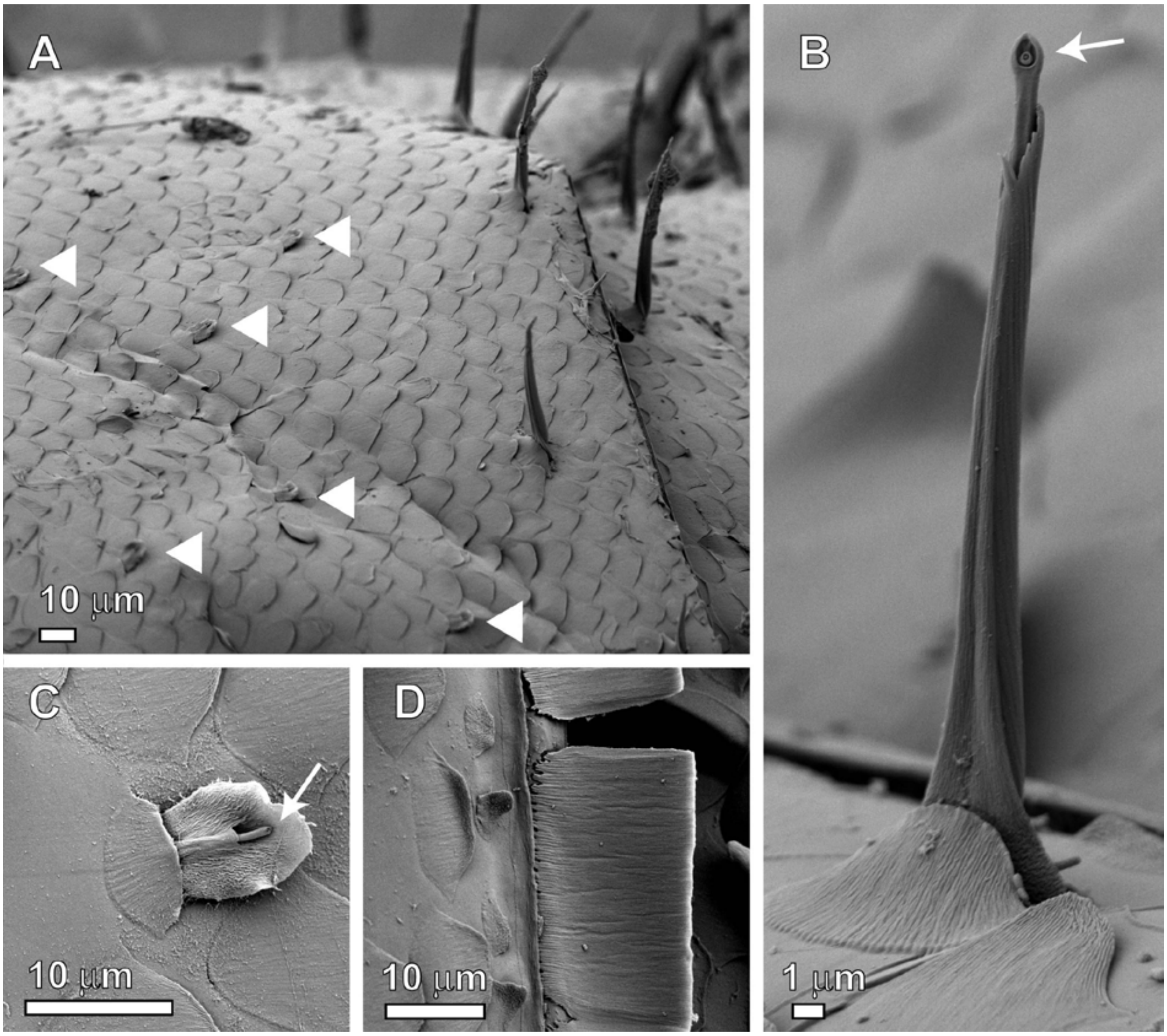


Fig. 5. Scanning electron micrographs of sensilla with a preceding scale on the cuticular surface of *Atlantoscia floridana*. A) Short- (arrowheads) and long-type sensillum formed by a sensory hair surrounded by a protective structure, scattered over the surface of the 7th tergite. B) Long-type sensillum perpendicular to the tergite surface with an overlapping scale at the base. C) Detail of the cuticular surface showing scattered scales and a short-type sensillum. D) Flap-like scale at the posterior edge of the tergite. Arrows indicate the apical pores.

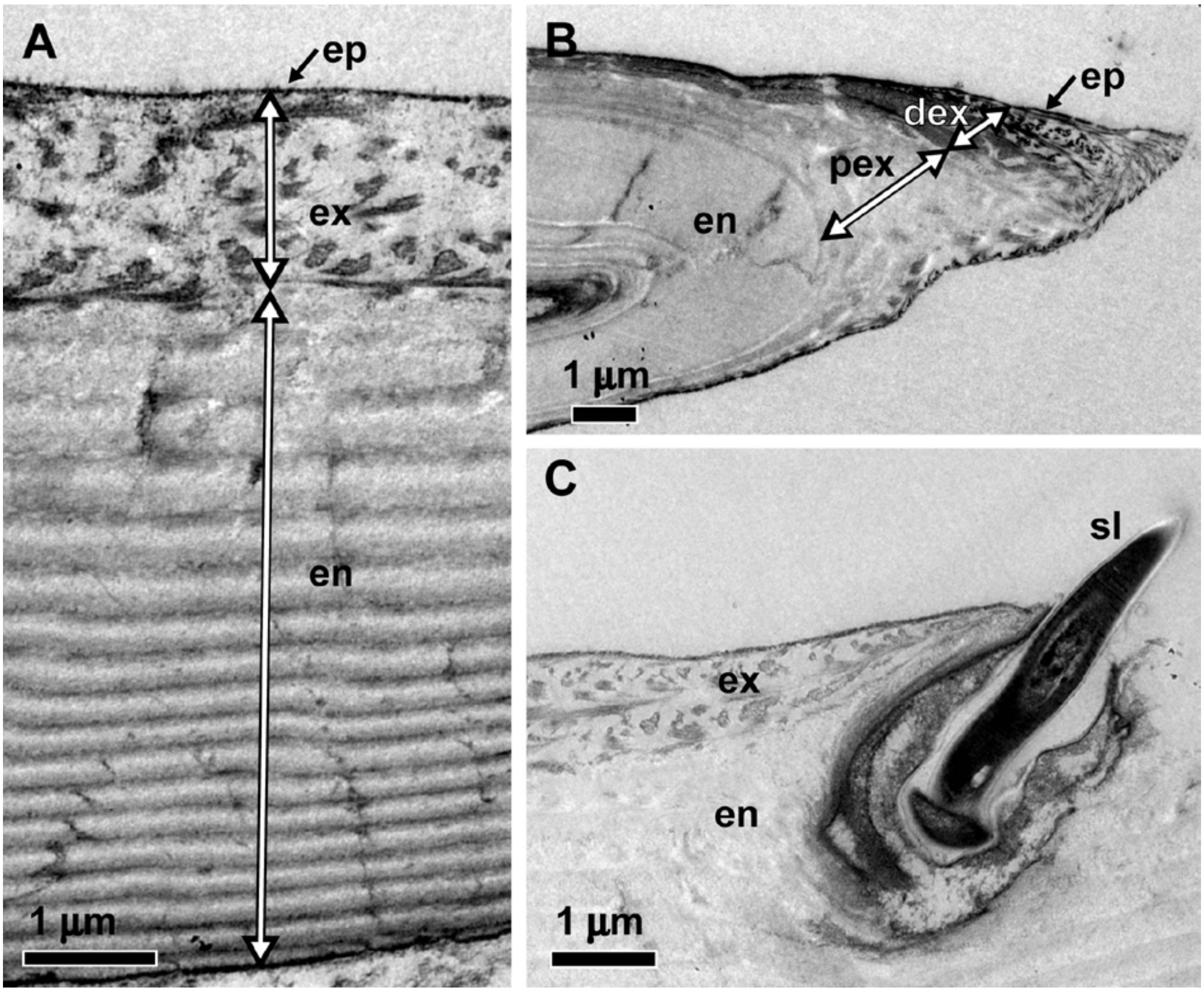
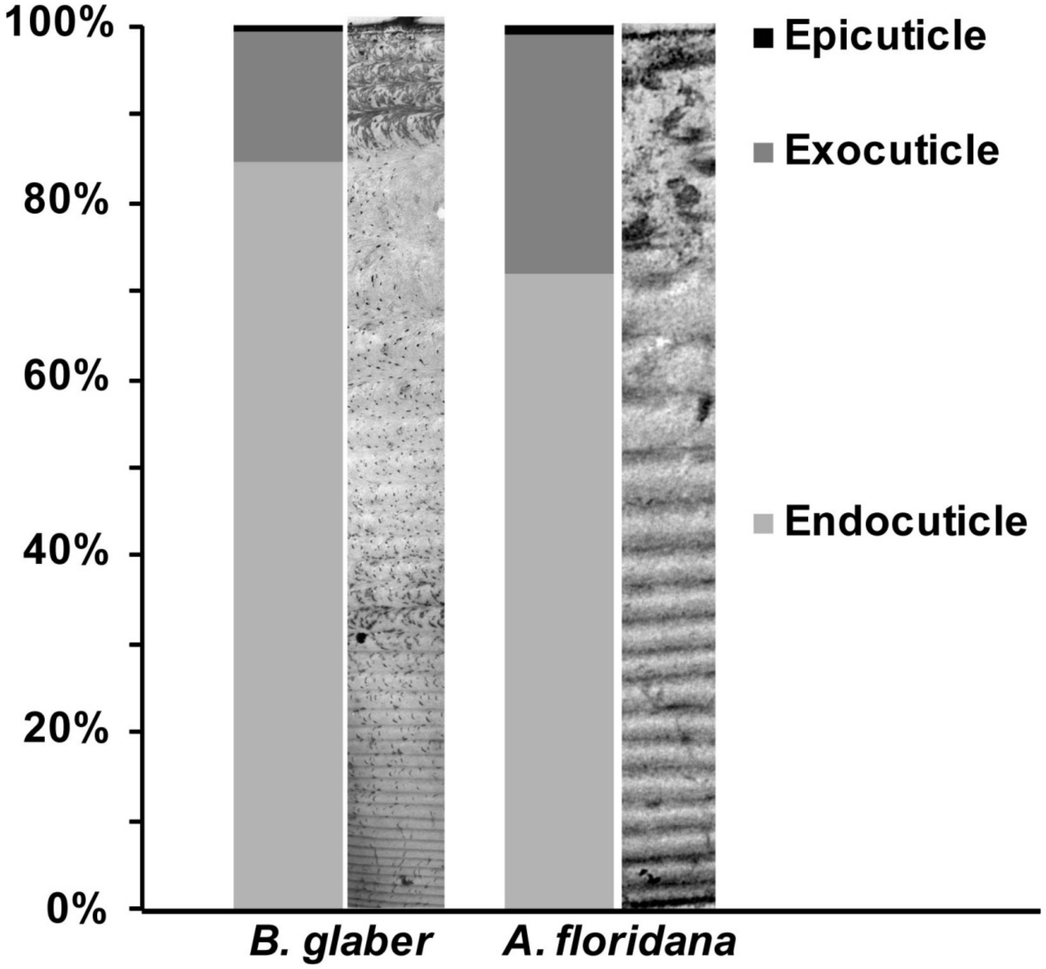


Fig. 6. Transmission electron micrographs of the cuticle structure of *Atlantoscia floridana*. A) Parasagittal section of the cuticle showing different layers. B) Posterior edge of the tergite showing the distinction of the proximal (pex) and distal (dex) exocuticle. C) Short-type sensillum partially cutting through the cuticle. ep, epicuticle; ex, exocuticle; en, endocuticle; dex, distal exocuticle; pex, proximal exocuticle; sc, scale; sl, sensillum.



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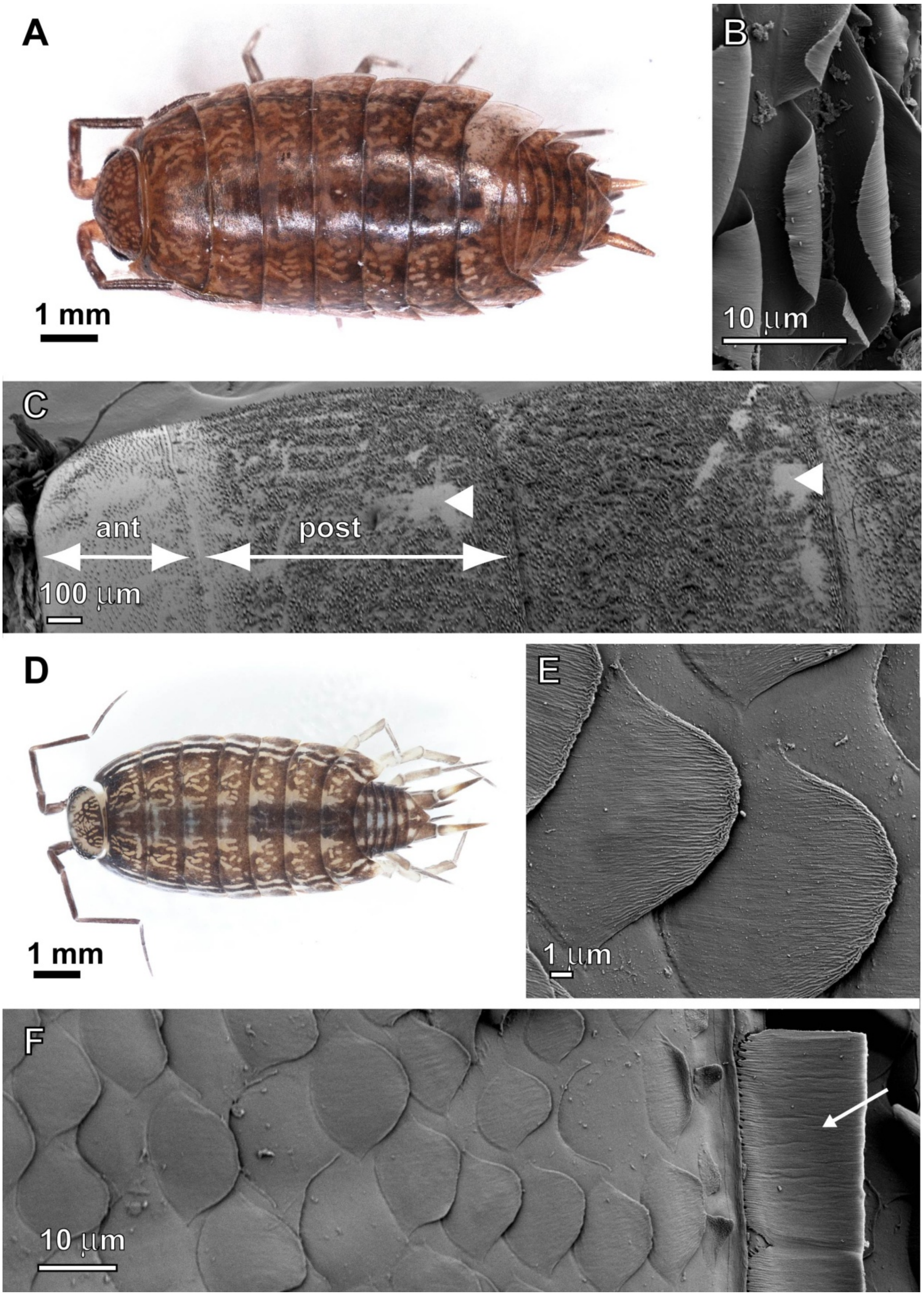

Supplementary material. Fig. Dorsal cuticular surface of *Balloniscus glaber* (A-C) and *Atlantoscia floridana* (D-F). A) General appearance of *B. glaber*. B) Scanning electron micrograph showing overlapping curved scales. C) Scanning electron micrograph of 5th, 6th and partially 7th tergite, showing dense coverage with curved scales and partially descaled areas on the exposed posterior surface of the tergite (post) (arrowheads) and less-scaled anterior part of the tergite (ant) covered by removed 4th tergite. D) General appearance of *A. floridana*. E) Scanning electron micrograph of flat semicircular scales. F) Scanning micrograph of a tergites surface covered with flat scales and flap-like scale at the posterior edge (arrow)

“Science never solves a problem without creating ten more”

George Bernard Shaw

(Irish playwright)

Capítulo II

Experimental evidence of dietary calcium influence on molt cycle and cuticular structure of two woodlice species (Crustacea, Isopoda, Oniscidea)

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Content:

Wood, C.T., Ziegler, A., Araujo, P.B. In prep. Experimental evidence of dietary calcium influence on molt cycle and cuticular structure of woodlice (Crustacea, Isopoda, Oniscidea).

Title Page

Artigo a ser submetido para o periódico Invertebrate Biology

Experimental evidence of dietary calcium influence on molt cycle and cuticular structure of two woodlice species (Crustacea, Isopoda, Oniscidea)

Running title: Dietary calcium and molting in woodlice

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Abstract.

Terrestrial isopods molt frequently throughout their lives in order to grow and/or renew surface receptors. The molting cycle in terrestrial isopods can be affected by external and internal factors and involves strategies to recycle body calcium such as biphasic molting. We used experimental units with artificial diet and structural analysis of the cuticle to understand how calcium-deficient and calcium-rich food sources interfere with the molt cycle of two species with different cuticular characteristics. Peculiarities such as lateral stretching of the antennae were observed during ecdysis of *Atlantoscia floridana* and might relate to specific morphology of the species. Intramolt duration was longer for *Balloniscus glaber* as well as overall survivorship in treatments. Cycle duration was influenced by calcium concentration. A trend of shorter molt cycle was observed with increasing calcium concentration for *B. glaber* while in *A. floridana* only difference between control and treatments was observed. Degree of mineralization of *B. glaber* showed negligible difference between treatments. Regardless of treatment and species, higher mortality rate under lab conditions seems to be related to the process of ecdysis itself with cumulative mortality of 20% from the beginning of posterior ecdysis until the beginning of postmolt period.

Key words: cuticle, ecdysial failure, experimental diet, exuviae, elemental distribution, mineralization degree

Introduction

Ecdysis is a periodic process of shedding the old cuticle by which arthropods grow. The rigid external cuticle of arthropods serves as an exoskeleton that does not allow continuous growth. Frequency of ecdysis is associated with body size (Lawlor 1976), so molt cycle duration increases with animals growth. Some species enter a period of anecdysis after reaching a certain size while others continue to molt for the entire life. Nonetheless, ecdysis is only one part of the molt cycle that involves resorption and deposition of a new cuticle before the old one is shed. In crustaceans, this molt cycle involves calcification that is the primary method by which they harden their cuticle (Greenaway 1985). Terrestrial crustaceans such as woodlice uptake calcium exclusively by the food, unlike other crustaceans that can assimilate calcium from the water (Ziegler 2003) and therefore need to recycle high amounts of calcium from the old cuticle prior ecdysis. Deposition of amorphous calcium carbonate in sternal deposits during premolt (Ziegler 1994), biphasic molting and consumption of exuviae (Greenaway 1985) are some strategies that allow woodlice to recycle about 50 % of the calcium (Steel 1993) from the old cuticle.

Detailed descriptions of the ecdysial process in woodlice have been published (Heeley 1941, Hatchett 1947, Paris & Pitelka 1962, Vittori et al. 2012) as well as data on molting frequency of different species (Edney 1964, White 1968, Lawlor 1976, Steel 1980, Zidar et al. 1998, Štrus & Blejec 2001, Vittori et al. 2012). Regarding morphological differences of the cuticle throughout the molt cycle, there are several studies with different species regarding the formation and structure of sternal calcium deposits (i.e. Wood & Russell 1987, Ziegler 1994, Vittori et al. 2012), cuticle deposition (i.e. Messner 1965, Steel 1982, 1993, Štrus & Compère 1996, Ziegler 1997) and quantification and distribution of minerals (i.e. Becker et al. 2005, Hild et al. 2008, Seidl et al. 2011, Neues et al. 2011). However, other than season (Paris & Pitelka 1962) and temperature (Edney 1964, Steel 1980), little is known about environmental factors that interfere with the molting cycle. Regarding nutrition, it has been shown that metal contamination decreases molt frequency (Drobne & Štrus 1996) while food deprivation didn't affect drastically molt cycle duration (Heeley 1941, Drobne & Štrus 1996).

Although one would expect that animals require an external calcium source to mineralize a new and larger cuticle with the same degree of mineralization, only few studies acknowledge the effects of external calcium on woodlice. Steel (1993) described the storage and translocation of calcium during the molt cycle in the integument of *Oniscus asellus* Linnaeus, 1758 and reported that the slower calcification that occurs at postmolt, stage that likely requires intake of dietary calcium to complete calcification. Regarding consumption, one study using four sympatric terrestrial isopod species showed that a plant species with high calcium content (10.57% by weight) was readily eaten by all four isopod species (Dudgeon et al. 1990), while a more recent work analyzed several litter traits and showed that preference increases with increasing leaf calcium content among other leaf traits (Quadros et al. 2014). Concerning addition of calcium, a study using a supralittoral isopod species showed increased consumption for calcium carbonate enriched algae (Pennings et al. 2000) and a microcosm experiment showed higher growth rates and lower risk of mortality of terrestrial isopods in limed conditions (Tompson et al. 2013). Nonetheless, these studies do not relate the effect of external calcium on the molt cycle.

This study aims to understand how calcium-deficient and calcium-rich food sources interfere with molt cycle, growth and degree of mineralization of woodlice.

We hypothesize that the lack of enough dietary calcium can either interfere with molt cycle duration and/or cuticular degree of mineralization. *Atlantoscia floridana* (Van Name, 1940) and *Balloniscus glaber* Araujo & Zardo, 1996 are two species with different cuticular characteristics and from different ecomorphological groups that are commonly found in sympatry. Therefore, they were chosen to observe the effect of varying dietary calcium on molt cycle aspects under laboratory conditions.

Material and Methods

Source-site, species and laboratory culture

Atlantoscia floridana and *B. glaber* are two isopod species commonly found is sympatry in southern Brazil. Collections in two locations (30°4'10.19" S, 51°7'10.19" W and 30°20'2.50" S, 51°16'57.82" W) were carried out to establish laboratory culture. Animals were captured in the field with the aid of forceps and taken to laboratory. Soil temperature was recorded at the time of each collection and an average temperature was used in the culture chamber.

Species were sorted and kept in plastic containers with soil and leaf litter from the collection sites. Males and females were kept in different culture chambers in order to decrease the number of breeding females. The animals were maintained at 17 ^{o}C and 12:12 photoperiod. Medium size adults were used in the experiments with an average cephalothorax width (CW) of 1.05 ± 0.17 mm for *A. floridana* and 1.89 ± 0.21 mm for *B. glaber* (for growth curves see Araujo & Bond-Buckup 2004, Meinhardt et al. 2007). Both species were kept in culture for at least one month prior to the beginning of experiments. This period allowed animals to stabilize molt cycle to culture conditions since this transition frequently triggers ecdysis.

Experimental units and treatments

Each week, culture units were sorted and adult premolt males and non-breeding females were separated in individuals units (Ø= 54 mm). The animals were kept with moistened cotton and one leaf from culture until ecdysis of the anterior half of the body. The day of the anterior ecdysis was recorded (beginning of postmolt) and the leaf was replaced by experimental food.

Experimental food modified from Lardies et al. (2004) was made in laboratory. A pilot study was conducted to verify acceptance of food by both species and to determine the proportions of the ingredients in the diet. The control consisted of 50 % corn starch, 16 % peptone, 25 % cellulose, 1 % vitamin mix, and 8% lipid (vegetal oil). Ingredients were measured and mixed, and distilled water was added to the mixture to complete 20 mL. The solution was transferred to a clean petri dish and placed in oven at 40 °C until dry. The treatments consisted of the same basic mixture with the addition of 1, 4 or 8 % of calcium carbonate to it provided that leaf species with more than 8% of calcium content are not common in the litter. Experimental food was kept in isolated dishes at the same culture chamber for up to one month. After this time, new food was prepared. Remaining food from units was replaced every second weeks Animals were observed every other day, and the day of anterior and posterior ecdysis was recorded. Signs indicating imminent ecdysis such as the presence of sternal calcium deposits or whitish coloration in one half of the body, consumption of exuviae and specific behavior related to molting process were also recorded. Manipulation of animals was avoided in the days before and after ecdysis.

Cephalothorax width of animals was measured (*sensu* Sutton 1968) at least two days after the posterior ecdysis since animals are soft and need some time to expand and harden the cuticle. For the measurement, pictures of animals were taken in stereomicroscope SMZ800 (Nikon) and CW was measured using ImageJ Software (Abràmoff et al. 2004). New units were made to replace animals that died during the first ecdysis after individualization until each treatment had a sample number of at least 10. Animals that presented signs of bacterial infection or presence of parasitoids were excluded from the analysis. Average duration of molt cycle and percentage of animals that survived a full molt cycle was calculated. Survivorship was compared between species, treatment and treatment within the species using a mixed linear model on SPSS software (IBM) version 18.

Mineralization of the cuticle

For each treatment, 15 new units with *B. glaber* were made. Animals were kept with experimental food for 15 days after completion of anterior ecdysis. After this time, animals were sacrificed by freezing and dissected under stereomicroscope. Tergites 2 and 6 were washed with absolute methanol, air dried and store under -20 °C for elemental analysis.

Electron microprobe analysis

Methanol-treated dry tergites were cut sagittally and glued on aluminum holders with the middle part exposed. Samples were first platinum coated to increase conductivity of the sample and then polished. Polishing was done in a Ultracut ultramicrotome (Leica, Austria) using a 45º diamond knife (Diatome, Switzerland) advancing into the cuticle by 90, 70, 40 and 20 nm for at least 15 times each (Fabritius et al. 2005). Samples were then carbon coated and electron microprobe analysis was performed with a Zeiss 962 scanning electron microscope (Oberkochen, Germany) equipped with a Falcon (EDAX) X-ray detector system using GENEIS software. The microscope was set to 20 kV acceleration voltages and 10 µA emission current in analysis mode. Aluminum standard was used to adjust count rate and hence normalize electron beam intensity. Elemental maps were recorded at a 256 x 200 pixel resolution with a full spectrum recorded and stored for each pixel. For quantification, samples were embedded in glue prior to coating and polishing to remove calcium signal obtained from the tergal surface.

Since cuticle thickness is correlated to body size (Vittori & Štrus 2014), net intensity and molar percentage values were corrected by average CW. For that, average CW of 2.0 was estimated from the growth curve of *B. glaber* (Meinhardt et al. 2007) and therefore used to normalize data. Normalized Ca and Mg net intensity values were used as a measurement of degree of mineralization.

Field emission scanning microscopy

After elemental analysis, tergite samples were cleaved for structural observation. Samples were rotary coated were with a 4 nm thick platinum layer (BAF 100, Balzers) and then observed with a Hitachi S-5200 field emission scanning electron-microscope (FESEM) at an acceleration voltage of 4 kV and an emission current of 10 or 20 µA.

Results

Ecdysis: general remarks

The shedding of the exuviae in both species involves ecdysial movements by the animal that continues, however at a lower rate, after animal has shed that half of the exuviae completely. These movements consist of expansion and relaxation of the body that makes the anterior half move forwards while the posterior half moves backwards. During this process, each tergite expands individually on the molting half while the non-molting half moves as a single block (Supplementary material).

Two peculiarities regarding the molting process not previously described on the literature were observed for *A. floridana*. Typically, the animal shifts its balance to the anterior pereopods while shedding the posterior exuviae. In *A. floridana*, we observed a few individuals stretching laterally the antennae at this stage (Fig. 1A). Also, it was noticed an overlap of the 6th and 7th pairs of pereopods over the uropods in this species (Fig. 1C). Neither strategy (lateral stretching of the antennae or overlap of pereopods) was observed for *B. glaber.* For both species, no difference in the molting process was observed between the begging of the experiment (before being subjected experimental diet) and after.

Ecdysis duration and exuviae consumption

The posterior and anterior ecdysis of *A. floridana* usually occurs within 24 h. On three occasions, the anterior ecdysis started without complete shedding off of the posterior half (Fig. 2A). All those occasions resulted in death of the individual: animals had failed to conclude posterior ecdysis after 1 or 2 days when they started molting the anterior half. Death also frequently resulted from impossibility of removing the antennae from the exuviae (Fig. 2B) or incapability of releasing the antennae that would sometimes glue together (Fig. 2C) or to the moist, freshly molted body. Regarding exuviae consumption, *A. floridana* ingests the anterior or sometimes the posterior exuviae, but only after the anterior ecdysis has finished and no intramolt exuvial consumption was recorded.

For *B. glaber*, anterior ecdysis usually begins 2 days (mean and mode values) after posterior ecdysis has ended. The shortest and longest intramolt intervals recorded were one and four days, respectively. Consumption of the posterior exuviae was observed during intramolt period in many cases, and sometimes consumption of the anterior exuviae during postmolt (Fig. 3). Treatments with varying calcium carbonate concentrations (0, 1, 4 or 8% $CaCO_3$) didn't change mean or median values of ecdysis duration or exuvial consumption for either species.

Survivorship on treatments

The average treatment longevity for *A. floridana* was around 19 days for all treatments. For *B. glaber*, the average treatment longevity was lower for treatments 0 and 1 % and higher for treatments 4 and 8 % (Table 1).

Significant difference in survivorship was observed between species ($F_{1,84} = 15.94$; $p < 0.001$) and no significant difference was observed among treatments ($F_{3,84} = 0.44$; $p = 0.723$) or among treatments within the species ($F_{3,84} = 0.58$; $p = 0.629$). Nonetheless, a difference in trend can be observed separating 0-1 % (low calcium) and 4-8 % (high calcium) treatments for *B. glaber*. A second analysis grouping low and high calcium treatments increased homogeneity of variances and showed significant difference among treatments ($F_{1,36} = 10.13$, $p = 0.003$) for *B. glaber* (Fig. 4).

Molt cycle duration on treatments

Molt cycle duration varied between treatments, from 26 to 22 days on average for *A. floridana* (Table 1). The longest cycles recorded were on the 0 % treatment. The average molt cycle duration was higher than the average treatment longevity. On each treatment, only 20 to 50 % of the animals were able to withstand a complete molt cycle (Table 1). No increment in cephalothorax width could be noticed since most animals died during or shortly after ecdysis.

For *B. glaber*, survivorship followed the opposite trend as molt cycle duration. Average molt cycle duration varied from 60 (0% treatment) to 38 (8% treatment) days. Only 20 % of the animals stayed alive during an entire molt cycle in treatments 0 and 1 %, and 40 % of animals from the treatments 4 and 8 %. Although two animals began ecdysis at the end of the first molt cycle on treatments 0 and 1 %, only one animal could complete the whole process. On the treatment 4 %, all the four animals that started the ecdysis could complete it. Only on the 8 % treatment there were animals that could outlast more than one complete molt cycle. About 40 % of animals (five individuals) could start the first ecdysis, 23 % (three individuals) started the second ecdysis, and 8 % (one individual) could start the third ecdysis. No increment in size could be verified since all recorded differences were smaller than 0.05 mm (low accuracy from live animal measurements).

Mineralization degree

Survivorship on experimental units was low and infection with nematode larvae was observed in many units that were excluded from the experiment. Therefore, only few animals remained and one sample was selected to analyze mineral content except for 0 % treatment (no survivor). Elemental maps show Ca, Mg, P and S distributed throughout the cuticle (Fig 5). Calcium signal recorded from the cuticular surface was blocked with the embedding of the sample in glue (Fig. 6), thus allowing quantification of elements solely from the cuticle section. Relative degree of mineralization differed slightly between samples. In the same animal, values were higher for the anterior tergite on treatment 1 % and on the posterior tergite on treatments 4 and 8 %. Molar percentage of Mg, P and Ca showed little variation between treatments and tergite and some variation was observed for S between treatments (Table 2).

Ultrastructure

Average cuticle thickness of *B. glaber* ranged from 12 to 15 µm. Thickness normalized by average population CW was 12-13 µm for treatment 1%, 14-15 µm for treatment 4% and 16-17 µm for treatment 8%. The anterior tergite of the 8% treatment presented the highest values although thickness was less constant throughout the tergite. In all samples, epicuticle, exocuticle, endocuticle and membranous layers can be identified (Fig 7).

Molt stage related survivorship (and life table)

Survivorship data was rearranged regarding molt cycle stage so that the beginning of posterior ecdysis indicates day 0. Hence, the number of days from the beginning of the last ecdysis was used rather than the total survival period on the treatment. For this curve, animals that died during the first postmolt were included. Survivorship fitted a sigmoid (polynomial) function with $R^2 = 0.96$ for *A. floridana* and $R^2 = 0.98$ for *B. glaber*, illustrating a typical type III survivorship curve with high initial mortality rate followed by a period of much lower, relatively constant mortality (Fig. 8).

About half the animals died within nine days after beginning of ecdysis for *A. floridana* and within 15 days for *B. glaber*. The highest daily mortality was 24 % registered for day 1 in *A. floridana*. Similar cumulative rate was observed for *B. glaber* around the 4-5th day (Table 3).

Discussion

This study used experimental laboratory conditions to control dietary calcium of two woodlice species with different cuticular characteristics in order to understand possible implications of external calcium on molt cycle. We observed that the species with thicker cuticle (i.e. *B. glaber*) was affected by dietary calcium concentration: molt cycle duration and mortality seem to be associated with dietary calcium while degree of mineralization is not. This thicker cuticle coupled with the observed effect of dietary calcium in molt cycle length indicate higher calcium requirement for *B. glaber* in comparison with *A. floridana*. We also observed that both species present higher mortality associated with ecdysial failure, although it is not known if this mortality rate relates to lack of proper ecdysial sites on experimental units or if the same rate of ecdysial failure happens on natural conditions.

Ecdysis: process and duration

Most of the observed ecdysis process is in agreement with previously published literature (Heeley 1941, Paris & Pitelka 1962, Vittori et al. 2012). However, we observed peculiarities during ecdysis that might be related to specific body morphology. *Atlantoscia floridana* is a runner and has long pereopods and antennae, so the observed overlap of pereopods might help the exuviation process. Furthermore, the lateral stretching of the antennae might help in maintaining balance when support lays solely in the anterior part of the body. The resting position of the antennae does not seem to help in this balance. Hence, other peculiarities are expected to be found on animals with different morphology whose ecdysis process has not been described yet.

Ecdysis duration differed between species and might be associated with timing of cuticle reabsorption and deposition. Deposition of cuticular layers are related to molt cycle phase so that intramolt reflects a difference in development of both halves rather than an artificial pause (Price & Holdich 1980a). Timing of deposition of specific layers is therefore different in each body half (Messner 1965, Steel 1982, 1993, Ziegler 1997), and it is retarded on the anterior half (Ziegler 1997). Nonetheless, the endocuticle and membranous layers are only secreted after molting of that half. *Atlantoscia floridana* presents thinner and more delicate cuticle than *B. glaber* (Wood et al. 2017) and is especially fragile during molt. As the number of endocuticular stacks in the cuticle of *A. floridana* is lower than in *B. glaber*, it should need less time to secrete the new endocuticle, possibly explaining the shorter intramolt duration. On the other hand, *B. glaber* has thicker cuticle with higher stack number and longer time for cuticle secretion is expected, explaining its longer intramolt duration. Other isopod species have been shown to have intramolt periods of 2 to 3 days on average while others molt both halves within 24 h (Heeley 1941, Hatchett 1947, Edney 1964, Messner 1965, White 1968, Zidar et al. 1998, Vittori et al. 2012). These studies usually report only one intermolt interval although involve more than one species, making it difficult to relate intramolt duration and cuticle thickness of other species.

Another factor that may help to explain the observed difference in intramolt duration between species is the environmental tolerance of each species. During ecdysis, the individual becomes more susceptible to desiccation and more subjected to unfavorable changes in microclimate (Paris & Pitelka 1962). *Atlantoscia floridana* is less resistant to environmental fluctuations than *B. glaber* as indicated by its higher population fluctuation throughout the year (Araujo & Bond-Buckup 2005). Therefore, it could benefit from faster intramolt duration that also decreases the time that the individual is even more vulnerable to desiccation.

Dietary calcium, molt cycle and cuticle

Experimental evidence indicates influence of dietary calcium on molting frequency of adult woodlice. This influence was not pronounced for *A. floridana*, although cycle was slightly longer in calcium deprived treatment. The thinner and more delicate cuticle observed on this species (Wood et al. 2017) coupled with its shorter molting cycle indicates its lower requirement for external calcium. On the other hand, the different trends for molt cycle duration and longevity on treatment of *B. glaber* suggests influence of low (0 and 1 %) and high (4 and 8 %) dietary calcium for this species. *Balloniscus glaber* has thicker cuticle with numerous endocuticullar stacks and probably has higher calcium demands than *A. floridana*. It has been previously suggested a lack of influence of feeding in molting frequency, since animals molt when under starvation (Heeley 1941). However, experimental units with woodlice usually contain plaster or rocks to maintain humidity therefore providing an external source of calcium carbonate to animals. Plaster consumption by woodlice subjected to insufficient food source has been previously observed (Wood & Zimmer 2014) and can explain why food source has not been associated with molting frequency before.

Cycle duration was influenced by calcium concentration and shorter molt cycle in animals subjected to high calcium food indicate that premolt may be shortened or anticipated when high amount of external calcium is available. It has been previously suggested that isopods need an external calcium source to mineralize the anterior cuticle after ecdysis (Steel 1993). However, the uptake of mineral from external sources from late premolt to postmolt has been excluded (Ziegler et al. 2017), since animals do not feed during this period with the exception of occasional consumption of exuviae (Steel 1993). Recently, observations on mineral content in different body parts of the same animal at different molting stages indicated that the recycled amount of calcium has been previously underestimated and that the ingested exuviae can be indeed used as a mineral source (Ziegler et al. 2017). Granules of calcium have been previously recorded in small hepatopancreas cells of the supralittoral *Ligia italica* during premolt that are depleted in intramolt and postmolt, suggesting a role as a storage site (Štrus & Blejec 2001), although these granules are probably not related to accumulation from the ingested cuticle (Ziegler et al. 2017). Therefore, external calcium may be more important for accumulation prior ecdysis (i.e. during premolt) for molting rather than for mineralization after ecdysis (i.e. during postmolt).

Plaster has been used in experiments with terrestrial isopods provided that it helps to maintain more constant humidity for animals. Eliminating plaster or other external calcium sources from experimental units makes it possible to control calcium availability to animals at the expense of possibly increasing fluctuations in humidity. Changes in humidity can affect survivorship on treatments thus making it difficult to gather enough data for more robust statistical analysis. Lower stability on experimental units could have interfered with longevity of animals, especially for *A. floridana* that is thought to have lower tolerance to environmental fluctuations.

The lack of noticeable increment in size after molting suggests that animals molt not only for growth. Hatchett (1947) and Heeley (1941) have observed that molting does not always result in measurable increase in size, so molting frequency in woodlice has also been associated with renewal of cuticular structures and sensory organs (Price & Holdich 1980b). *Atlantoscia floridana* possesses many long perpendicular sensilla that are easily broken upon animal manipulation (Wood et al. 2017). This species presented shorter molting cycles than *B. glaber* that has more robust surface sensilla. Hence, we suggest that the physical external environment can also be a factor that influences molt frequency in terrestrial isopods provided that the space for animals changes in different layers.

However molting frequency seems to be related to dietary calcium in *B. glaber*, the structure and degree of mineralization of the cuticle do not seem to be affected by the experimental diet. Besides calcium being recorded in both the exocuticular and endocuticular layers, there was calcium signal from the tergal cuticular surface. In crustaceans, the epicuticle itself is not mineralized, but the epicuticular surface can be impregnated with calcium salts (Travis 1955, Roer & Dillaman 1984). In *B. glaber* this calcium impregnation seems to also occur on the tergal surface. Regarding the degree of mineralization, there were only small differences between samples, not relating to treatment itself. This species either clings, runs or present tonic immobility in response to a predator attack, so it would be more advantageous to prolong the time for calcium accumulation prior ecdysis than to decrease degree of mineralization of the cuticle. The amount of calcium and magnesium from the tergal cuticle of *B. glaber* is lower than the values obtained for most of the isopod species studied for elemental aspects so far (*Armadillidium vulgare* (Becker et al. 2005), *Porcellio scaber* (Becker et al. 2005, Seidl et al. 2011), *Tylos europeus* (Seidl et al. 2011), *Oniscus asellus, Philoscia muscorum, Porcellio spinicornis* and *Armadillo officinalis* (Neues et al. 2007)). This difference might be associated with differences in the ecophysiology of the species, size, molting stage or experimental conditions.

Ecdysial failure

Regardless of treatment, higher mortality rate under lab conditions seems to be related to ecdysis itself. The survivorship curve obtained from the beginning of ecdysis show a rapid initial mortality rate followed by a lower and more constant rate. For *A. floridana* that molts both halves within 24 h, mortality rate was over 20 % at day 1. Similar cumulative mortality for *B. glaber* was recorded for around day 4 or 5 that has a 2 or 3 days gap between posterior and anterior ecdysis. Cumulative mortality of about 20 % was recorded around the beginning of postmolt period for both species. Therefore, this higher mortality period can be associated with the process of ecdysis itself. Failure of ecdysis can be attributed to a number of causes including failure to split the old cuticle or to expand body parts soon after ecdysis, although few data is available on this topic (Reynolds 1980). In crustaceans, most of what is known about this molt death syndrome is regarding economically important species from aquaculture, such as the cultured lobsters of the genus *Homarus* (Bowser & Rosemark 1981). For terrestrial arthropods, few studies mention this phenomenon and usually relate to chemicals that disrupt cuticle deposition or hormonal regulation (Leopold et al. 1985, Kabir et al. 2013). A couple of studies involving observation of woodlice mention death of isopods with the anterior part of the exuviae partially removed (Hatchett 1947). Regarding environmental factors, one study identified incomplete ecdysis as one of the major types of mortality during emergence of a dragonfly species on the field, and related the high incidence of this event with overcrowding and competition for ecdysial sites (Corbet 1957).

Nothing about ecdysial sites has been reported for woodlice besides the observation from Hatchett (1947) that individuals ready to molt seek a protected location on a rough surface. However, this behavior may rather relate to avoidance of cannibalism and predation during the time the animal remains soft and sluggish. Cannibalism has been occasionally recorded on woodlice and can be associated with horizontal transmission of bacteria (Bouchon et al. 2008, Le Clec'h et al. 2013). Regardless of the reason, this search for protected locations during ecdysis makes it difficult to find cases of ecdysial failure on the field, since animals would be easily crushed and less available for collection. Therefore we suggest that the frequency of this event on the field might be similar to frequency observed on laboratory conditions or which attributes an ecdysial site for woodlice would have. Future experiments using different substrates might help elucidate this question.

Future directions

This study presents experimental evidence on the effect of an external calcium source on molt cycle duration of woodlice. Nonetheless, several questions were raised from the information here presented and from the observation of animals during the experiments. Are there other peculiarities during the ecdysis process adopted by other species with different morphology that can help exuviation? Is intramolt duration related to cuticular thickness and deposition? Is premolt shorter if high amount of calcium is available of calcium? Why do animals consume the exuviae at different times? Can molting related to renewal of surface receptors be influenced by compaction of the surrounding environment? Is mortality rate due to ecdysial failure similar on the field? Do woodlice have and compete for ecdysial sites? Do these sites help the exuaviation process or just help to avoid cannibalism and predators? What attributes does an ecdysial site for woodlice have? Are they the same for different species? A lot questions are still to be answer relating to environmental factors affecting molting on terrestrial isopods.

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Table 2. Molar percentage of Mg, P, S and Ca and degree of mineralization (Ca+Mg net intensity) on a polished surface of the 2nd (anterior) and 6th (posterior) tergite of *Balloniscus glaber* fed on experimental diet with different calcium carbonate concentrations. Values are normalized by average cephalothorax width.

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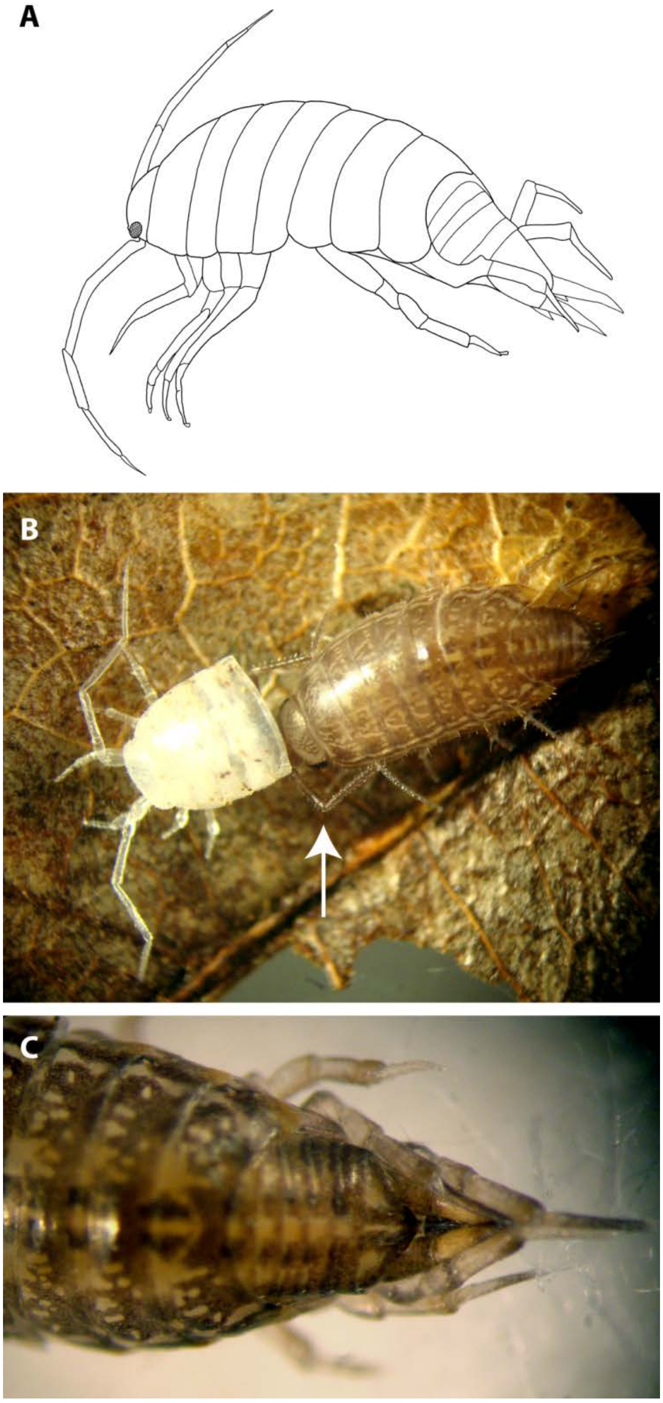

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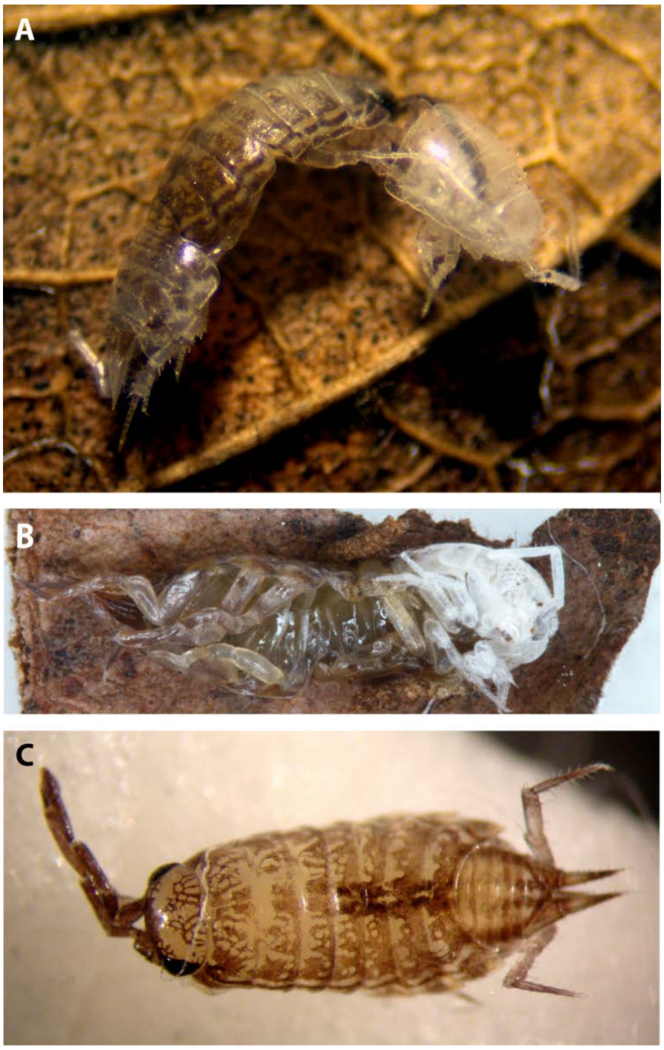

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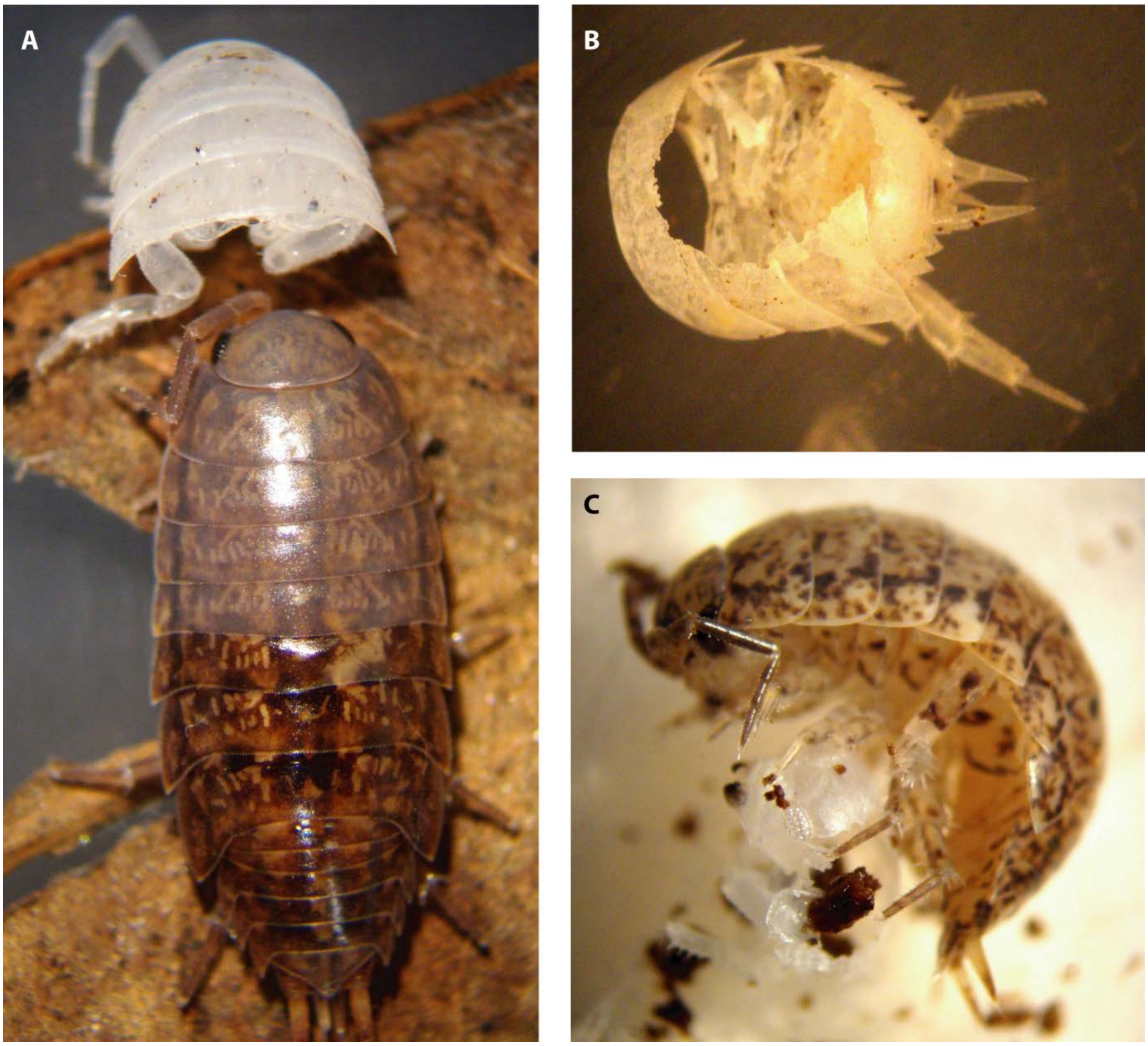

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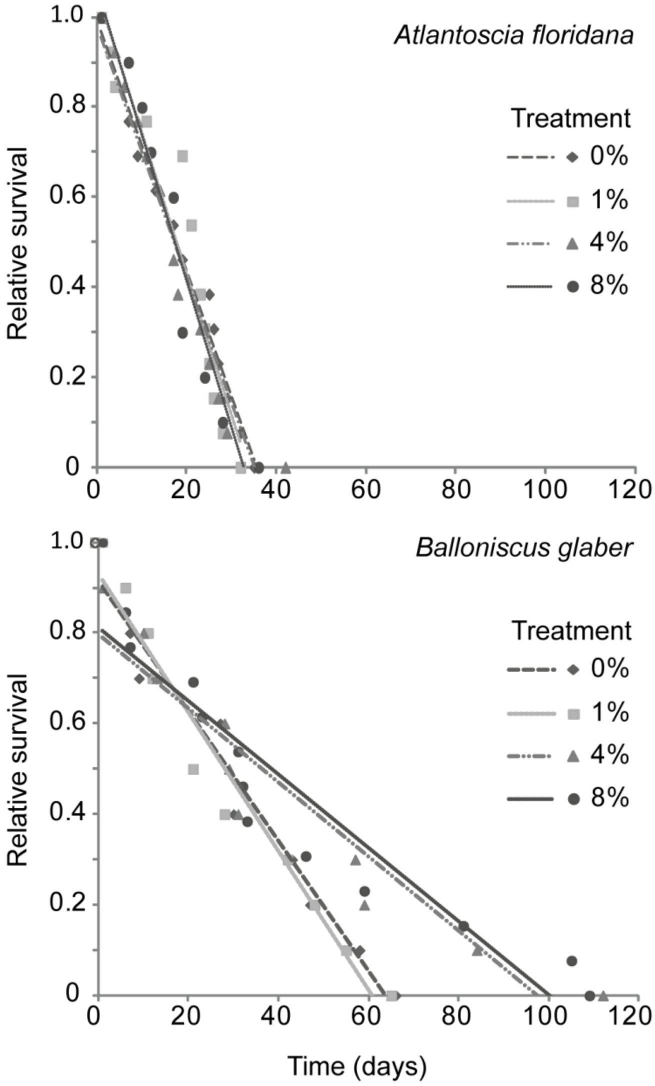

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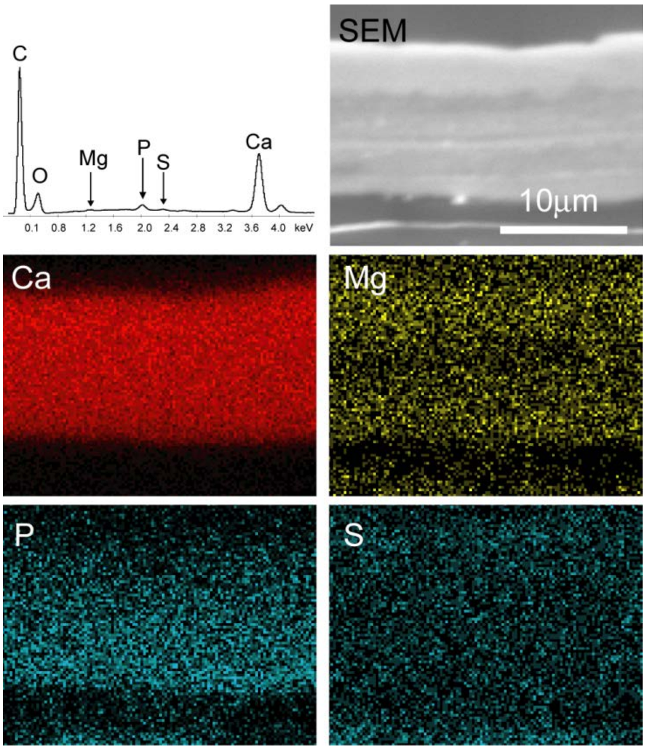

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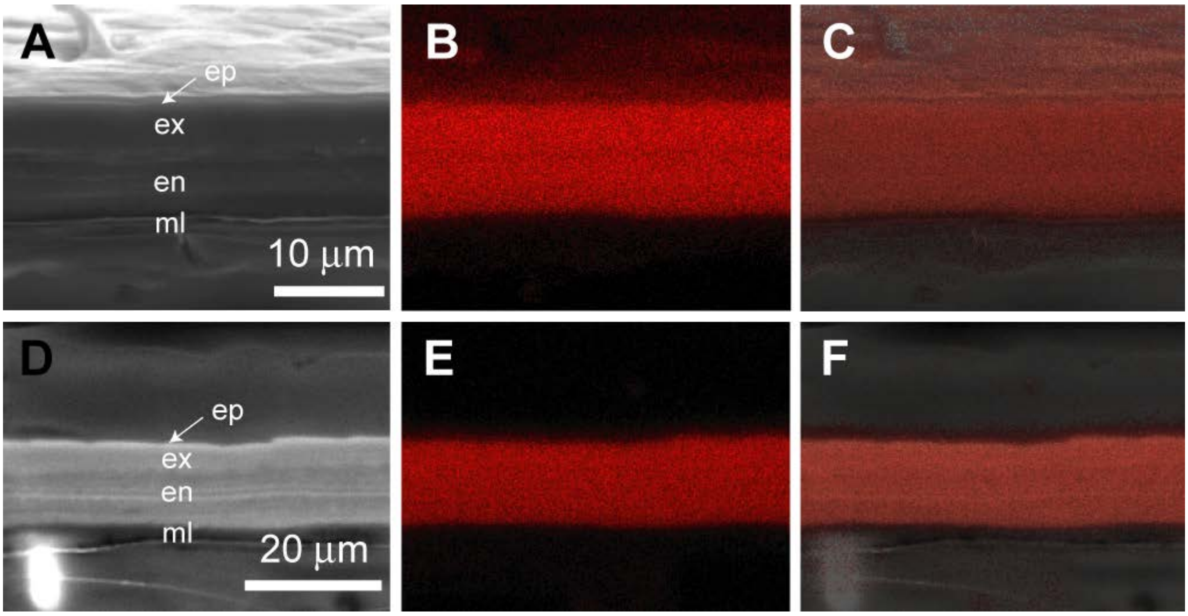

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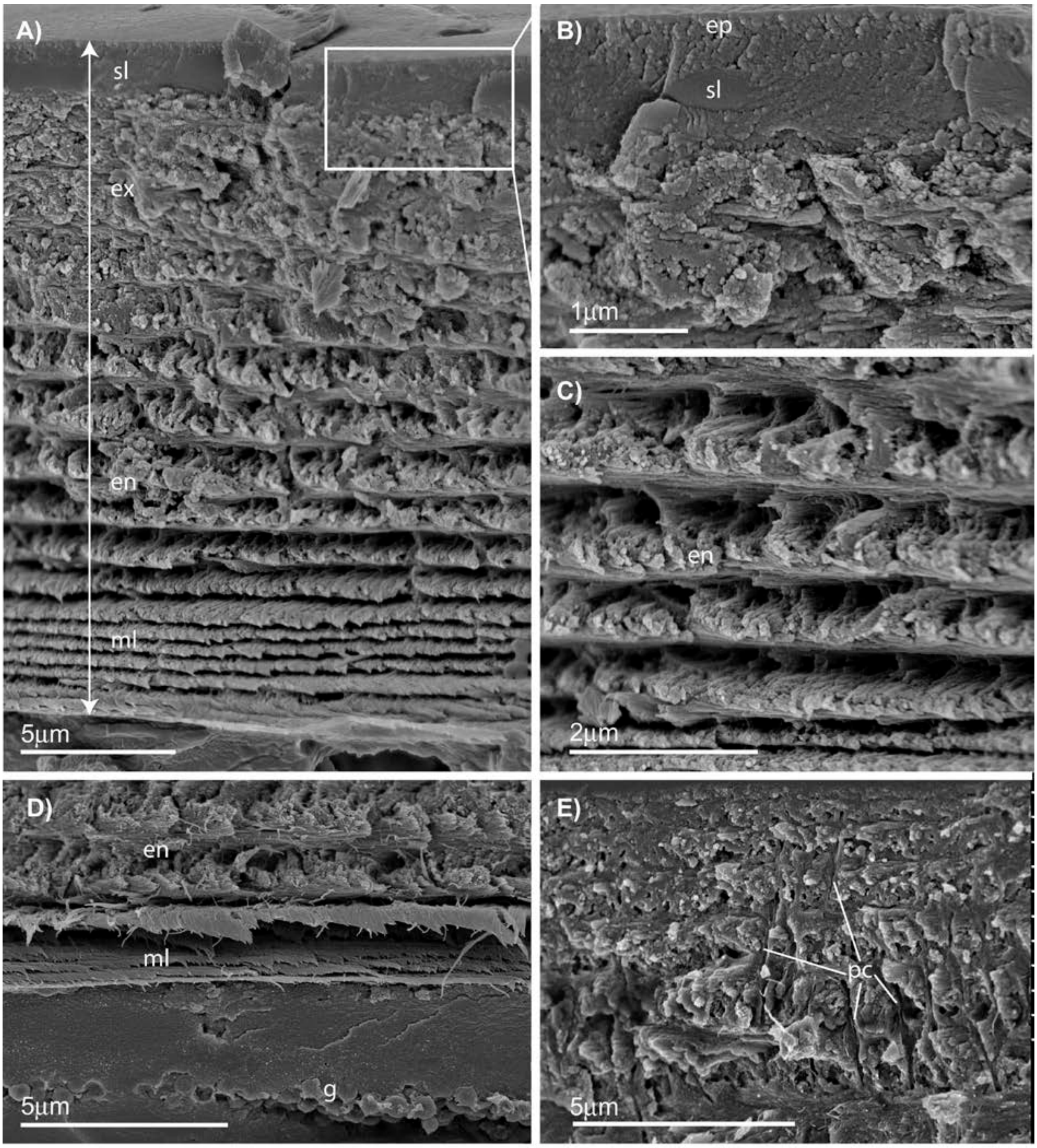

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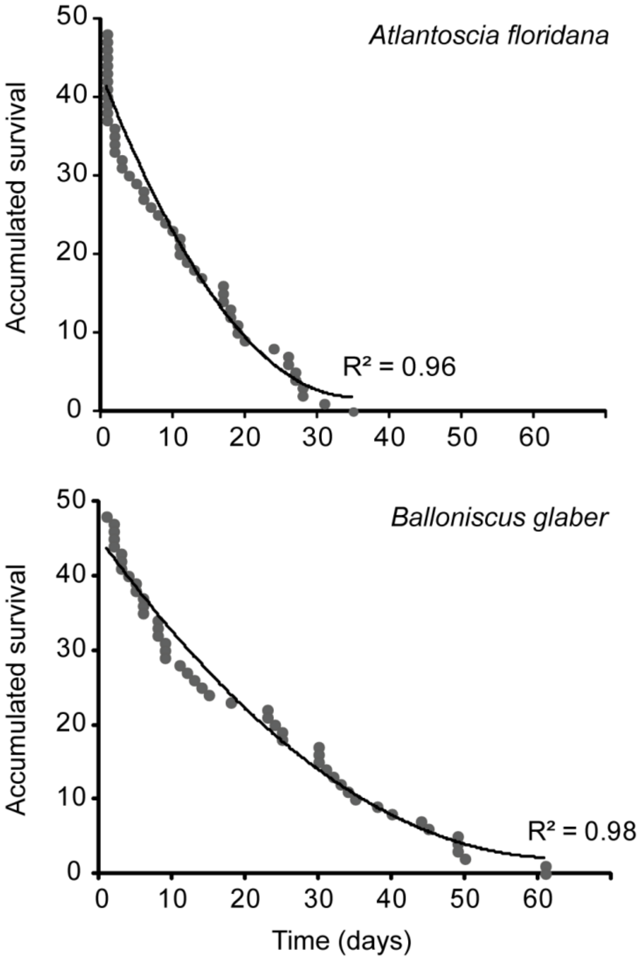

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Tables

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	Atlantoscia floridana				*Balloniscus glaber*			
Treatment	N	Survivorship	1st molt cycle		N	Survivorship	1st molt cycle	
			N (%)	Duration			N (%)	Duration
0%	13	19.2 ± 3.0	3 (23%)	26.3 ± 1.8	10	32.4 ± 6.7	2 (20%)	60.5
1%	13	20.0 ± 2.4	5 (39%)	21.8 ± 1.0	10	30.9 ± 6.4	2 (20%)	59.0
4%	13	18.6 ± 2.8	3 (21%)	22.0 ± 3.2	10	42.4 ± 11.2	4 (40%)	43.5 ± 8.0
8%	10	19.1 ± 2.7	5 (50%)	22.8 ± 2.9	13	43.0 ± 9.9	5 (38%)	37.6 ± 2.8
	49	19.2 ± 1.4	16 (31%)	22.6 ± 1.3	43	37.6 ± 4.4	13 (30%)	46.2 ± 3.8

Table 2. Molar percentage of Mg, P, S and Ca and degree of mineralization (Ca+Mg net intensity) on a polished surface of the 2nd (anterior) and 6th (posterior) tergite of *Balloniscus glaber* fed on experimental diet with different calcium carbonate concentrations. Values are normalized by average cephalothorax width.

Treatment	Body half	Mg	P	S	Ca	Degree of mineralization (net intensity)
1%$CaCO_3$	Anterior	0.14	0.47	0.03	5.49	195.8
1%$CaCO_3$	Posterior	0.11	0.33	0.06	4.35	166.3
4%$CaCO_3$	Anterior	0.13	0.51	0.08	5.30	191.5
4%$CaCO_3$	Posterior	0.14	0.52	0.10	5.20	200.3
8%$CaCO_3$	Anterior	0.14	0.52	0.17	4.50	184.3
8%$CaCO_3$	Posterior	0.13	0.60	0.24	5.17	209.4

Table 3. Relative and cumulative mortality rate of *Atlantoscia floridana* and *Balloniscus glaber* on laboratory conditions. Day 1 marks the beginning of ecdysis of the last molt cycle.

	Atlantoscia floridana		*Balloniscus glaber*	
Time (days) *x*	Mortality at day *x*	Cumulative mortality rate	Mortality at day *x*	Cumulative mortality rate
1	0.24	0.24	0.02	0.02
2	0.08	0.33	0.08	0.10
3	0.04	0.37	0.06	0.16
4	0.02	0.39	0.02	0.18
5	0.02	0.41	0.04	0.22
6	0.04	0.45	0.06	0.29
7	0.02	0.47	0.00	0.29
8	0.02	0.49	0.06	0.35
9	0.02	0.51	0.06	0.41
10	0.02	0.53	0.00	0.41
11-15	0.12	0.65	0.10	0.51
16-20	0.16	0.82	0.02	0.53
21-25	0.02	0.84	0.10	0.63
26-30	0.12	0.96	0.06	0.69
31-35	0.04	1.00	0.10	0.80
36-40			0.04	0.84
41-45			0.04	0.88
46-50			0.08	0.96
51-61			0.04	1.00

“Somewhere, something incredible is waiting to be known”

Carl Sagan

Capítulo III

Ultrastructural features of exoskeletal cuticle of two terrestrial isopod species (Crustacea: Oniscidea) during pre- and postmolt

Camila T. Wood, Jasna Štrus, Paula B. Araujo

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Ultrastructural features of exoskeletal cuticle of two terrestrial isopod species (Crustacea: Oniscidea) during pre- and postmolt

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ABSTRACT

Terrestrial isopods molt frequently throughout their lives and therefore they permanently degrade and renew their cuticle. This dynamics of resorption and deposition is affected by the biphasic molt and calcium deposition on sternal deposits prior ecdysis in terrestrial isopods. Analysis of cuticle deposition in *Atlantoscia floridana* and *Balloniscus glaber* during pre- and postmolt stages was done by transmission electron microscopy. Although *A. floridana* and *B. glaber* differ in overall cuticle structure, cuticle deposition during premolt in both species is similar as already described in other terrestrial isopod species. The new epicuticle in late premolt is very similar to the one in intermolt animals suggesting little or no postecdysial modifications of this layer. Electron dense granules observed in the ecdysial space during premolt suggest recycle calcium recycling. Based on morphology and size, these granules are possibly calcium granules further used for mineralization. Granules from the ecdysial space are deposited on the scales of the new epicuticle prior ecdysis, suggesting calcium impregnation on the epicuticle of these species. Anchoring junctions were observed in animals until late premolt since they need to expand the body during ecdysis. During postmolt, regions without typical lamellate cuticle indicate modification of the exocuticle after ecdysis since expansion and hardening must occur after ecdysis.

KEY WORDS: cuticle deposition; mineralization; integument; exoskeleton; woodlice

INTRODUCTION

Terrestrial isopods molt frequently throughout their lives resulting in constant cuticular resorption and deposition. Frequent molting in isopod species enables not only growth and reproduction, but also regeneration of cuticular surface structures of the integument (Price and Holdich, 1980a) and digestive tract (Štrus and Blejec, 2001; Štrus et al., 2008). The molt cycle is divided in several phases (Steel, 1982; Zidar et al., 1998) related to cuticle synthesis and mineralization (Travis, 1957; Messner, 1965; Steel, 1982) and involves complex hormonal regulation (Carefoot, 1993). Cuticle formation has been studied in several adult species of terrestrial isopods (Messner, 1965; Price and Holdich, 1980a, 1980b, Steel, 1982, 1993, Compère, 1990, 1995; Štrus and Compère, 1996; Ziegler, 1997; Štrus and Blejec, 2001; Vittori et al., 2012; Štrus et al., 2015) and, more recently, in embryonic stages as well (Mrak et al., 2012, 2015, 2017). Although differences are observed among them, the sequence of events during cuticle deposition follows a pattern typical for all studied species. The beginning of premolt is marked by an apolysis that separates the endocuticle from the underlying epidermis. Secretion of a new epicuticle and exocuticle occur prior ecdysis while the endocuticle and membranous layer are secreted after ecdysis. Therefore, the former layers are termed pre-ecdysial while the latter are postecdysial. The calcification of the exocuticle it thought to happen after ecdysis while the endocuticle is thought to be calcified during its secretion (Travis, 1955; Roer and Dillaman, 1984).

In terrestrial isopods, this dynamics of resorption and deposition is particularly interesting since animals present a biphasic molt. They first molt the posterior half of the body and then the anterior half with the division between the 4th and 5th tergite

(Heeley, 1941; Messner, 1965; Steel, 1982; Ziegler, 1997). Furthermore, they accumulate calcium in sternal deposits prior ecdysis as an adaptation to recycle calcium that is necessary to harden the cuticle (Greenaway, 1985). During premolt, calcium is resorbed from the posterior integument, stored in the anterior sternal deposits; epicuticle and exocuticle deposition occurs prior ecdysis and mobilization of these deposits for calcification of the new posterior cuticle follows (Ziegler, 1997). The interval between posterior and anterior ecdysis is called intramolt and relates to timing of deposition of cuticular layers rather than a real "break" (Price and Holdich, 1980b). During intramolt, deposition of the anterior cuticle is retarded (Ziegler, 1997). At this stage, the resorbed calcium from the anterior half is transported to the hemolymph and later used for calcification of the anterior cuticle after ecdysis (Ziegler and Merz, 1999). Besides being mobilized to the sternal deposits, granules of amorphous calcium carbonate are resorbed from the old cuticle and observed on the ecdysial space prior ecdysis. This calcium granules are thought to be transported to epithelial cells and/or intercellular spaces and then to the hemolymph and later used for calcification of the new cuticle (Ziegler, 1994; Štrus and Compère, 1996).

Many steps related to these ultrastructural changes are not yet known and each work adds on to the knowledge of this process. Furthermore, ultrastructural changes during postmolt have been explored to a lesser extent than premolt in terrestrial isopods. Therefore, this work aims to describe the ultrastructural changes related to cuticle deposition of two species of terrestrial isopods from different ecomorphological categories and with different overall cuticular structure in order to shed light on the dynamics of cuticle resorption and deposition at two different molt cycle stages.

MATERIAL AND METHODS

Animal collection and fixation

Specimens from *Atlantoscia floridana* (Van name, 1940) and *Balloniscus glaber* Araujo and Zardo, 1995 were sampled in two locations near urban areas of Porto Alegre, RS, southern Brazil (30°4'10.19" S, 51°7'10.19" W and 30°20'2.50" S, 51°16'57.82" W). The average cephalothorax width of *A. floridana* and *B. glaber* were 1.1 and 1.9 mm, respectively, being all animals medium sized adults. Animals in premolt (i.e. with sternal calcium deposits) and postmolt (i.e. after anterior ecdysis and with moist appearance on anterior part) were separated for ultrastructural analysis.

Animals were sacrificed by freezing and fixed in Karnovsky fixative (2.5 % glutaraldehyde, 2 % paraformaldehyde in 0.1 M Hepes buffer, pH 7.3) for 3 days at 4 °C. Samples were then washed in Hepes buffer prior to further steps. Anterior and posterior tergites were isolated and decalcified with EDTA in Hepes buffer solution at 4 °C for 4 hours for *A. floridana* and for 5 days for *B. glaber* (differences relate to overall animal toughness). After decalcification, samples were washed in Hepes buffer solution.

Transmission electron microscopy (TEM)

Samples were post-fixed in osmium tetroxide for one hour and washed in Hepes buffer solution. Dehydration in ascending ethanol series (ethanol 30 %, 50 %, 70 %, absolute, acetone), infiltration and embedding in Agar 100 followed. For that, samples were kept in agar with acetone overnight and agar-acetone mixtures were changed twice on the following day before the addition of accelerator. Polymerization was performed in embedding molds at 60 °C for 48 h.

Parasagittal sections were cut with Reichert Ultracut S ultramicrotome (Leica). Semithin sections (0.5 μm) were cut with a glass knife, stained with Azur II – Methylene Blue and photographed with a Zeiss AxioImager Z.1 light microscope, equipped with an HRC Axiocam camera and Axiovision software. Ultrathin sections (70 nm) collected on copper grids were contrasted with 4 % uranyl acetate and 10 % lead citrate and examined with a CM 100 transmission electron microscope (FEI). Micrographs were recorded with BioScan 729 and Orius 200 cameras (Gatan) and Digital Micrograph software.

RESULTS

As the sequence of ultrastructural events is the same on both posterior and anterior parts despite its asynchronous deposition due to biphasic molting (Compère, 1990), results from both parts are presented together.

Premolt

Apolysis can already be seen in animals that do not yet have sternal calcium deposits (Fig. 1B-C). This separation between membranous layer and epidermis is not visible in all parts of the tergite at once (Fig. 1A), but can be observed in both posterior and anterior tergites at early premolt. Detachment between membranous layer and epidermis was observed in some parts of the cuticle (Fig. 1B) while in other parts, detachment between lamellae of the same layer was observed (Fig. 1C). The proximal postecdysial lamellae become thinner and thinner as an electron dense layer appears on the proximal end of the cuticle facing the ecdysial space (Fig. 1D, 2B, E). Later, this electron dense layer merges with a fibrous granulated sheet (Fig. 1D).

The new epicuticle is secreted from the epidermis and appears initially as short electron dense protrusions. This layer is thin and discontinuously secreted; the small segments gradually fuse together forming a continuous layer at later stages. Scales (epicuticular protrusions) are thinner at the beginning of secretion than the corresponding structures in intermolt (Fig. 2 A). Electron dense granules at the basis of the old cuticle are more abundant on the ecdysial space at later premolt stages (not shown). Some granules appear still associated with the fibrous sheet while others appear without clear connection to the ecdysial space (Fig. 1D). Some of these granules are gradually deposited along the surface of the scales from the new cuticle (Fig. 1D-E, Fig. 2D). In some parts, these scales are in close proximity with the old cuticle (Fig. 2C). Epicuticle with scales is formed before beginning of secretion of new lamellar exocuticle (Fig. 2C-D).

Sites with electron dense bundles of fibers that extend throughout the ecdysial space into the old cuticle form apical anchoring junctions (Fig. 1F, 2F). These attachment sites can be observed until late premolt in both species.

Postmolt

After ecdysis, some parts of the tergite present all differentiated layers. At first, the endocuticle present fewer lamellae that are very thin at the proximal end (Fig. 3A). In these portions, the epicuticle is formed and the exocuticle consists of 3-5 formed lamellae. Near the proximal end of the cuticle, electron dense granules are present dispersed in a fibrous sheet close to the new endocuticle (Fig. 3B-C, H).

For *B. glaber*, no clear distinction of the layers is possible soon after ecdysis in some parts of the tergal cuticle. On these sites, the exocuticle appears as a matrix with 1 to 3 structured lamellae with large vesicles underneath this layer (Fig. 3 D-G). Endocuticular lamellae can be identified in regions close to the epidermis although less structured than in the formed endocuticle. Electron dense granules can be seen at the interface of exo and endocuticle (Fig. 3D-F) as well as at the proximal end of the cuticle, close to the epidermis (Fig. 3F, H). Pore canals with electron dense material are abundant at the proximal end of the new endocuticle (Fig. 3E). The same situation was not observed in *A. floridana.*

DISCUSSION

The present work provides information regarding the dynamics of cuticle resorption and deposition in *Atlantoscia* and *Balloniscus*. Details not previously described for these two species could be observed in this study such as the deposition of the granules from the ecdysial space along the surface of the newly formed scales during premolt. Furthermore, the typical ultrastructure of the layers during postmolt was not clearly observed in some parts of the tergite, suggesting that there is some reorganization of previously deposited layers after ecdysis.

Overall, cuticle deposition during premolt in *A. floridana* and *B. glaber* followed the same pattern as previously observed for other terrestrial isopod species (Price and Holdich, 1980b; Štrus and Compère, 1996; Ziegler, 1997; Štrus and Blejec, 2001; Štrus et al., 2003, 2015; Vittori et al., 2012) . The new epicuticle in late premolt is very similar to the one in intermolt (Wood et al., 2017) as observed for *Titanetes albus* (C. Koch) (Vittori et al., 2012), also suggesting little or no postecdysial modifications on this layer. Compère (1990) observed loose granular material setting down on the epicuticular surface during postmolt and suggested that this material had been discharged by numerous dermal gland ducts as on the cement layer of insects, layer that is not present in either the species here presented or on *T. albus*.

Electron dense granules observed in the ecdysial space during premolt suggest that *A. floridana* and *B. glaber* not only shift calcium to sternal deposits but also recycle calcium within the same segment. These granules appear first in close proximity with the endocuticle that is being resorbed and latter associated with a fibrous and granular sheet on the ecdysial space. The granules observed for both species resemble those identified in *Ligia italica* (Štrus and Compère, 1996; Štrus et al., 2003, 2015; Žnidaršič et al., 2010; Matsko et al., 2011) in size, shape and location. Those granules accumulated in the ecdysial space during premolt were identified as calcium granules that are thought to be later transported by hemolymph to mineralize the cuticle after ecdysis (Štrus and Compère, 1996; Štrus and Blejec, 2001).

Some granules from the ecdysial space seem to be deposited on the scales of the new epicuticle prior ecdysis. The epicuticle of *Ligia italica*, *Armadillidium vulgare* and *Porcellio scaber* is not mineralized (Štrus and Compère, 1996; Neues et al., 2007; Hild et al., 2008) although calcium salts have been shown to be impregnated on the surface cuticle of decapod crustaceans (Travis, 1955; Roer and Dillaman, 1984; Dillaman et al., 2005; Roer et al., 2015). Calcium was identified through mineral analysis on the surface of *B. glaber* although not quantified (Wood et al in prep – capítulo 2). It is possible that the granules deposited along the surface of the new scales are from other materials from the old cuticle rather than calcium.

Anchoring junctions similar to those described by Žnidaršič et al. (2012) were observed in animals until late premolt. During ecdysis, the tergites from molting half expand individually while the whole non-molting half moves as a single block (Wood et al., 2017). As animals need to expand the body during ecdysis in order to shed the exuviae, the maintenance of sites with muscular attachment are important and therefore observed until ecdysis.

During postmolt, regions without typical lamellate ultrastructure were observed, indicating modification of the exocuticle after ecdysis. As the new cuticle expands after ecdysis, hardening by tanning and calcification has to occur after its deposition. This expansion of the cuticle after ecdysis impose disruption on the pre-ecdysial lamellate structure (Price and Holdich, 1980b). This might explain regions were lamellate structure is highly affected during postmolt (Fig. 3D-G).

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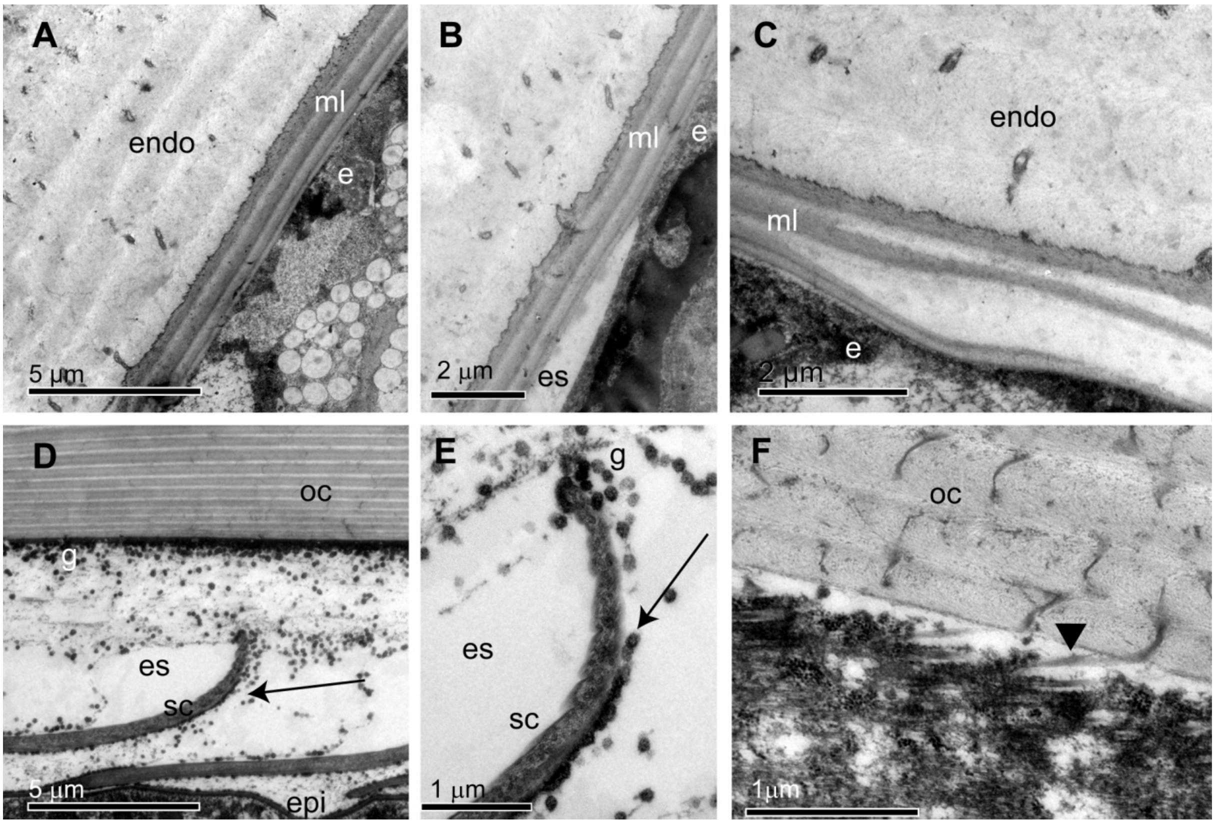

Fig. 1. TEM micrographs from the tergal cuticle of *Balloniscus glaber* during premolt. A) Early premolt stage with portions where the epidermis is still attached to the old cuticle. B) Site with detachment between membranous layer and underlying epidermis. C) Site with separation between lamellae of the membranous layer. D) Late premolt stage with fully formed scales and epidermis and with the presence of electron dense granules (arrow) in the ecdysial space. E) Detail of electron dense granules from the old cuticle being deposited (arrow) on the surface of a newly formed scale. F) Attachment sites with fibers extending into the old cuticle (arrowheads). **endo**, endocuticle; **ml**, membranous layer; **e**, epidermis; **es**, ecdysial space; **oc**, old cuticle; **g**, electron dense granules; **sc**, scale; **epi**, epicuticle.

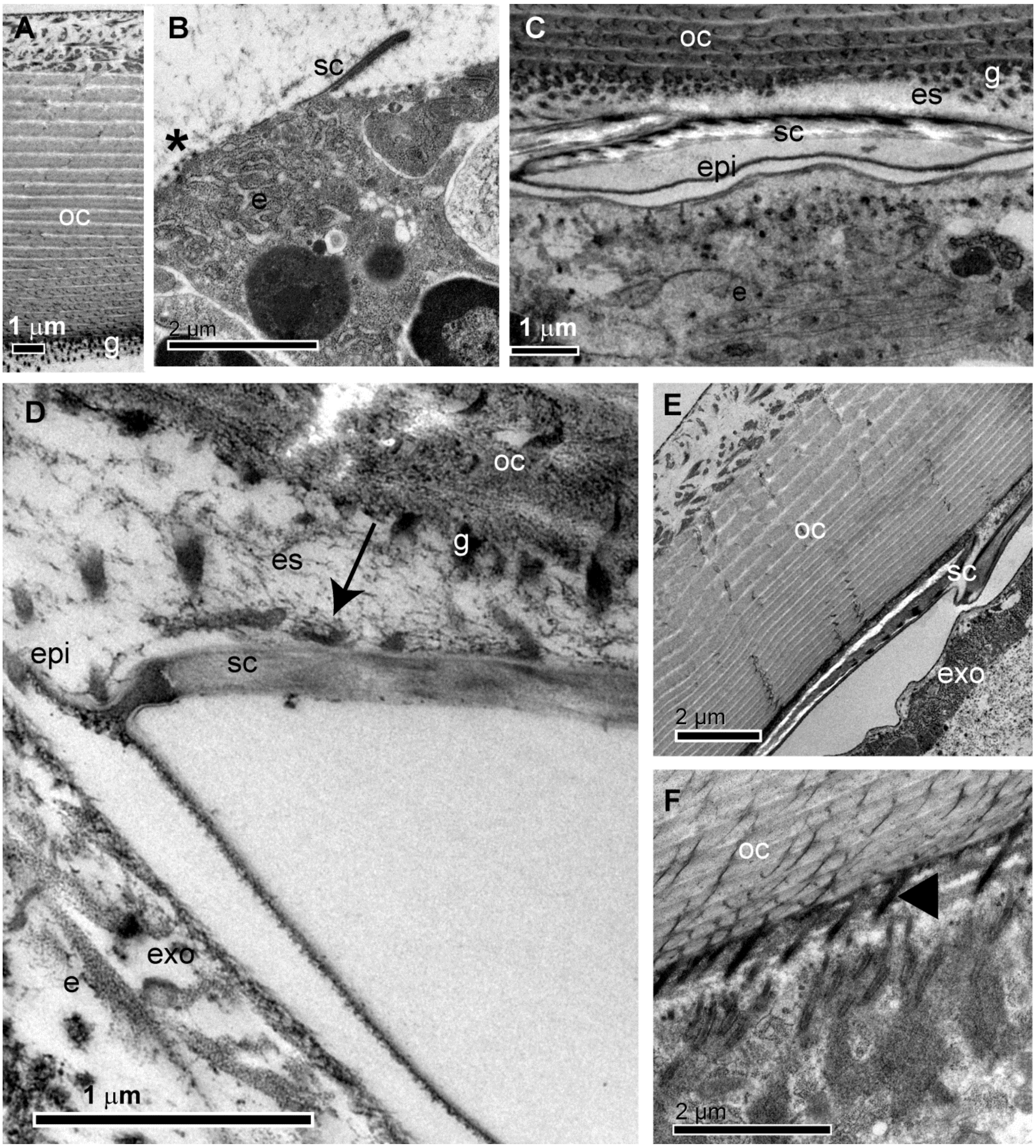

Fig. 2. TEM micrographs from the tergal cuticle of *Atlantoscia floridana* during premolt. A) Electron dense layer at the proximal cuticle with granules facing the ecdysial space. B) Early premolt stage with finger-like projections from the epidermis (*) and the beginning of secretion of the epicuticular scales by the epidermis. C) Premolt stage with formed epicuticle and scales and electron dense granules in the ecdysial space. D) Late premolt stage with formed epicuticle and scales and beginning of secretion of the exocuticle. Deposition of granules from the ecdysial space along the surface of the new scales (arrow). E) Scales in close proximity to the old cuticle. F) Attachment sites with fibers extending into the old cuticle (arrowheads). **oc**, old cuticle; **g**, electron dense granules; **e**, epidermis; **sc**, scale; **es**, ecdysial space; **epi**, epicuticle; **exo**, exocuticle.

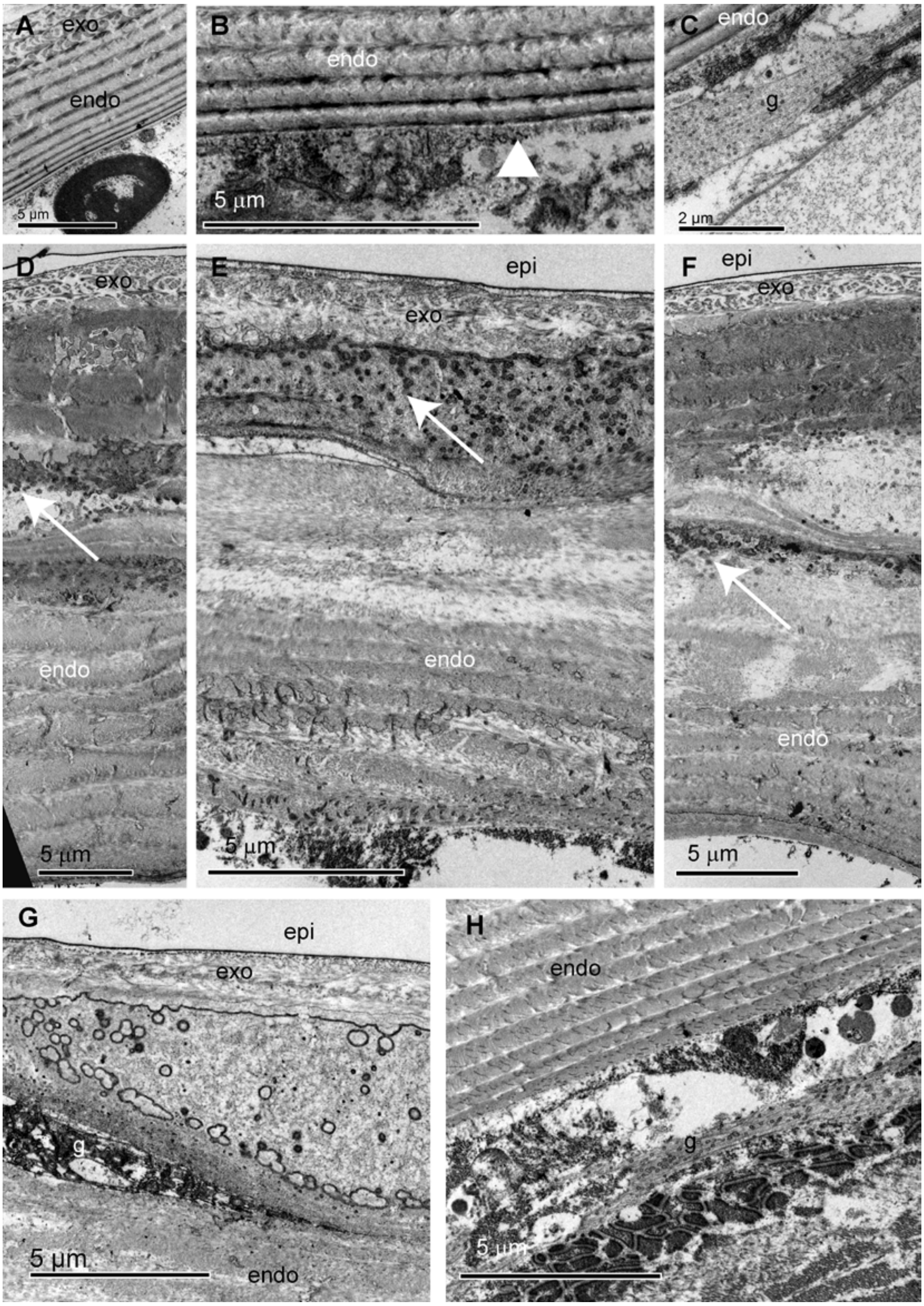

Fig 3. TEM micrographs of the tergal cuticle of *Balloniscus glaber* during postmolt. A-C) Deposition of the endocuticle (arrowhead) and presence of granules (g) within a fibrilar sheet reaching the new endocuticle. D-F) Partially structured cuticle with deposition of electron dense granules (arrow) in the exocuticle/endocuticle interface. G) Transport of material from electron dense granules (g) within the newly formed cuticle. H) Transport of material from electron dense granule from the epidermis to the proximal layers of the new endocuticle. **exo,** exocuticle; **endo**, endocuticle; **g**, electron dense granules; **epi**, epicuticle.

Considerações finais

A cutícula dos artrópodos é uma estrutura versátil que está ligada com o sucesso ecológico do grupo em colonizar diferentes ambientes terrestres e aquáticos. Além disso, a mesma é usada como inspiração para biopolímeros uma vez que as modificações estruturais observadas em diferentes espécies e partes do corpo possuem distintas propriedades mecânicas.

Os capítulos dessa tese abordaram as características estruturais da cutícula, a influência do cálcio alimentar no ciclo de muda e na mineralização e a dinâmica de reabsorção e deposição da cutícula de duas espécies neotropicais de isópodos terrestres. Os dados aqui apresentados ajudam a explicar relações entre as características ecomorfológicas e a história de vida (capítulo I), entre fonte de cálcio externa e ciclo de muda (capítulo II) e contribuiu com novas informações relacionadas à dinâmica de secreção cuticular no ciclo de muda (capítulo III). Entre os principais resultados e conclusões, esse estudo esclareceu os seguintes pontos:

- ✓ a superfície dos tergitos de *B. glaber* apresenta escamas curvas que podem estar relacionadas com a diminuição da força de adesão da água na superfície do corpo
- ✓ as sensilas de *B. glaber* são robustas e orientadas caudalmente, possibilitando o hábito endógio da espécie;
- ✓ a ultraestrutura da cutícula de *B. glaber* com grande espessura total e número de lamelas sugere altas propriedades mecânicas que são condizentes com a estratégia anti-predatória de aderente. Essa característica cuticular também

pode estar associada a maior tolerância ambiental encontrada nessa espécie que possui menor flutuação populacional relacionada a variações sazonais;

- ✓ as sensilas longas e perpendiculares a superfície do corpo encontradas em *A. floridana* impossibilitam o hábito endógio. No entanto, essa disposição pode favorecer o tempo de resposta anti-predatória uma vez que este prolongamento aumenta a superfície de percepção do ambiente;
- ✓ a fileira de escama na margem posterior dos tergitos de *A. floridana* pode constituir em uma barreira física para prevenção de entrada de partículas indesejadas no espaço intersegmentar;
- ✓ a ultraestrutura da cutícula de *A. floridana* com menor espessura total e número de lamenlas endocuticulares e fibras da exocutícula mais frouxas indica que a cutícula dessa espécie possui natureza leve, condizente com o hábito de corredora. Essa cutícula também pode estar relacionada a menor tolerância ambiental da espécie que é mais afetada por variações sazonais;
- ✓ *Atlantoscia floridana* apresenta posturas específicas tais como sobreposição dos pereópodos 6 e 7 sobre os urópodos e alongamento lateral das antenas que possivelmente ajudam a exuviação e manutenção do equilíbrio durante a ecdise;
- ✓ motivos ligados a falha ecdisial em *A. floridana* incluem começo da ecdise anterior antes de terminar a ecdise posterior, incapacidade de remover as antenas da exúvia ou desgrudá-las uma da outra ou do corpo que fica úmido pelo fluído ecdisial;
- ✓ a ecdise posterior e anterior geralmente ocorre dentro de 24h para *A. floridana* e com um intervalo de 2 dias para *B. glaber*. Essa diferença pode estar relacionada à espessura total da cutícula e ao tempo de secreção das camadas cuticulares durante a intramuda;

- ✓ *Atlantoscia floridana* pode consumir a exúvia apenas na pós-muda enquanto *B. glaber* pode consumir a exúvia tanto na intra- e pós-muda. Essa diferença pode estar relacionada a duração do período de intramuda ou da mineralização da cutícula nessa fase;
- ✓ a concentração de cálcio alimentar pode influenciar a duração do ciclo de muda e a sobrevivência de isópodos. O efeito do cálcio alimentar pode ser sido anteriormente subestimado devido ao uso de gesso em unidades experimentais que constituem uma fonte de cálcio externa para os animais;
- ✓ *Atlantoscia floridana* é menos afetada do que *B. glaber*. Essa diferença pode ser devido ao menor requerimento de cálcio dessa espécie que possui uma cutícula mais fina e flexível;
- ✓ há uma tendência a diminuição do ciclo de muda e aumento da sobrevivência em *B. glaber* com o aumento da concentração do cálcio alimentar. O cálcio alimentar pode ser mais importante para o acúmulo de cálcio na pré-muda do que na mineralização na pós-muda;
- ✓ ecdise sem crescimento notável sugere que existam outros fatores além do crescimento tais como a renovação de estruturas da superfície, e que o ambiente externo pode interferir com a frequência da ecdise;
- ✓ o grau de mineralização da cutícula de *B. glaber* não foi afetado pela concentração de cálcio alimentar. O cálcio está presente na exocutícula e endocutícula bem como impregnado na superfície embora não esteja presente na epicutícula ou na camada membranosa.
- ✓ elevadas taxas de mortalidade em condições de laboratório indicam relação com a fase da ecdise. Uma mortalidade acumulada de 20% dos indivíduos de ambas as espécies foi registrada entre o início da ecdise posterior e final da ecdise

anterior/início da pós-muda. Essa taxa de falha ecdisial pode ser similar em campo;

- ✓ a sequência de secreção e absorção da cutícula nas duas espécies é semelhante apesar das diferenças ultraestruturais encontradas na cutícula madura;
- ✓ grânulos provenientes da cutícula velha são armazenados no espaço ecdial durante a pré-muda e/ou depositados na superfície das escamas da epicutícula nova. Esses grânulos possuem morfologia semelhante aos grânulos de cálcio acumulados no espaço ecdisial em *Ligia italica* sugerindo que parte do cálcio seja reciclado diretamente da cutícula velha para a nova antes da ecdise;
- ✓ regiões pouco estruturadas na cutícula de *B. glaber* na pós-muda podem estar relacionados a reorganização estrutural em algumas partes da cutícula devido a expansão do tergitos durante o processo de ecdise.

Apesar do presente estudo esclarecer algumas questões, várias outras perguntas surgiram deste trabalho que podem ser futuramente estudadas. Como são as propriedades mecânicas da cutícula nos animais vivos? Qual a função dos diferentes tipos de sensila? Como as escamas de *B. glaber* podem ajudar a reduzir as forças adesivas da água? Que outras peculiaridades no processo de ecdise estão associadas à morfologia específica? A duração da intramuda está relacionada à deposição da cutícula nova? Por que os animais consumem a exúvia em momentos diferentes? Que gatilhos estão envolvidos na indução da muda? Pode a quebra de um grande número de receptores externos ser um dos gatilhos? A mortalidade relacionada à falha na ecdise em condições naturais é similar a encontrada em condições de laboratório? Quais os atributos relacionados a sítio ecdisial para isópodos terrestres? Os animais competem por sítios ecdisiais? Como o espaço ecdisial possibilita que ocorra ao mesmo tempo a absorção da cutícula velha e deposição da cutícula nova? Como é feito o transporte de diferentes constituintes da cutícula durante a síntese? Além disso, diversas lâminas histológicas e micrografias de transmissão (como a figura a seguir) obtidas nesse trabalho ainda estão sendo analisadas, e talvez algumas delas ajudem a esclarecer outros pontos que ainda não foram abordados.

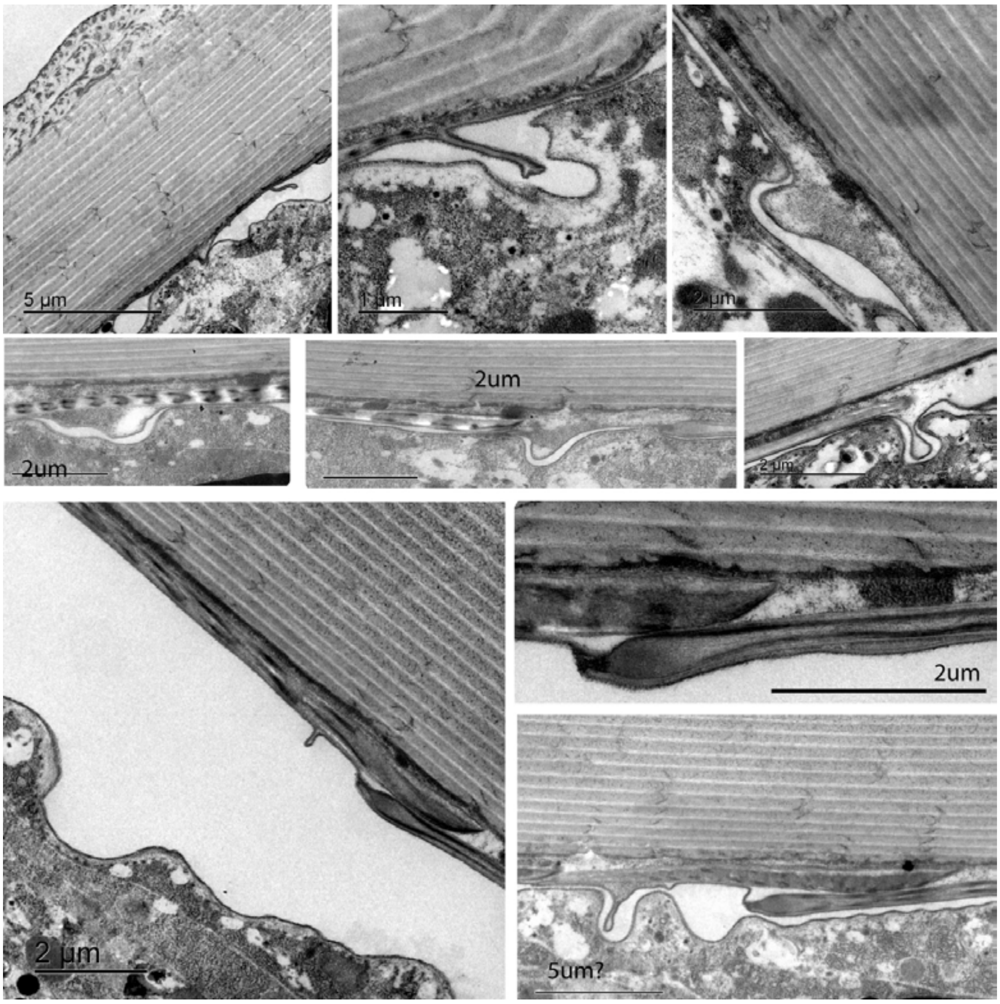

Fig 1. Micrografia de transmissão de *Atlantoscia floridana* na pré-muda tardia.

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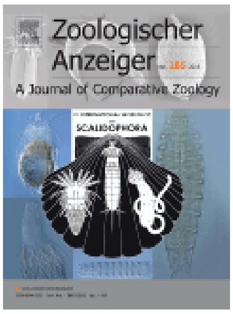

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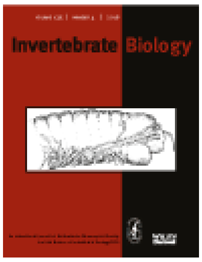

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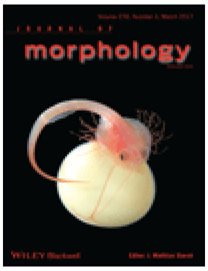

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