

Changes in cuticle structure during growth in two terrestrial isopods (Crustacea: Isopoda: Oniscidea)

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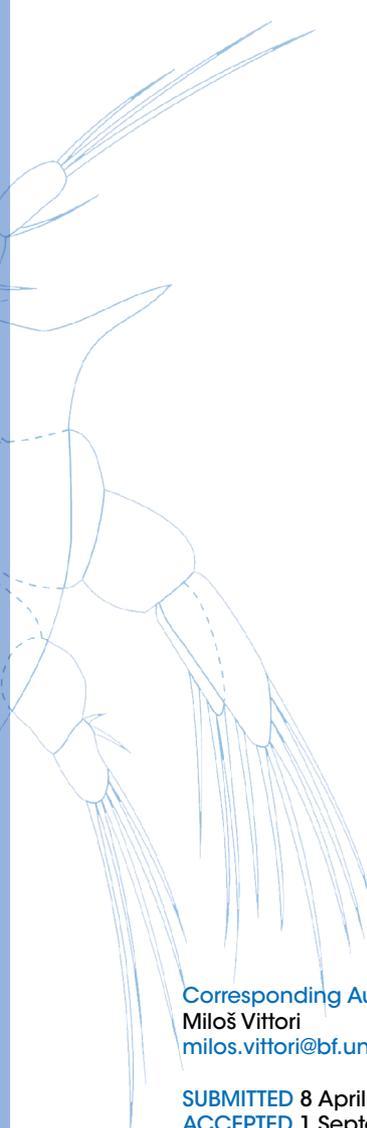
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ABSTRACT

Terrestrial isopods are a successful group of terrestrial crustaceans. Their exoskeletal cuticle has been studied in several species. However, it is not known whether the cuticle of these animals becomes thicker or how its structure changes as the animals grow. We aimed to determine the principles of upscaling of the exoskeletal cuticle during growth in the terrestrial isopods *Armadillidium vulgare* (Latreille, 1804) and *Porcellio scaber* Latreille, 1804 with scanning electron microscopy. The tergal cuticle becomes thicker with increasing body length. In *A. vulgare*, which rolls into a ball in defense against predators, the rate of increase of tergite thickness was greater than in *P. scaber*, which clings to the substrate when threatened. As the cuticles of both species become thicker, the proportion of the endocuticle in the cuticle increases. There is a strong correlation between cuticle thickness and the thickness of endocuticular lamellae. This indicates that in thicker cuticles, chitin-protein fibers in sequential layers change their orientation by a smaller angle. We found no significant differences in morphometric parameters between the cuticles of *A. vulgare* and *P. scaber* when controlling for cuticle thickness. This suggests that known differences in cuticle structure and composition between these two species may result from differences in cuticle thickness.

KEYWORDS

Development, exoskeleton, morphometry, ultrastructure, woodlice



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INTRODUCTION

The cuticle covers the bodies of arthropods and forms their exoskeleton. In crustaceans, it is often not only sclerotized but also mineralized with calcium carbonates and calcium phosphates. This makes it a natural composite material with chitin-protein fibers embedded in a mineralized matrix.

Several layers are distinguishable in the crustacean cuticle (Roer *et al.*, 2015). The epicuticle is a thin surface layer consisting predominantly of proteins and forming various scales and other surface ornaments (Schmalfuss, 1978; Price and Holdich, 1980) and containing only minute amounts of mineral (Seidl and Ziegler, 2012). The remainder of the cuticle is divided into the exocuticle, located immediately beneath the epicuticle, and the underlying endocuticle. An additional, non-mineralized innermost layer — the membranous layer — can be identified in some crustaceans (Hild *et al.*, 2008). Its thickness is variable and it may be completely absent in some individuals, even in species in which it is recognizable (Vaugh *et al.*, 2009; Wood *et al.*, 2017).

In the crustacean cuticle, chitin-protein fibers change their direction in a helicoidal sequence — the Bouligand pattern — as the cuticle is deposited (Bouligand, 1972; Raabe *et al.*, 2005; Roer *et al.*, 2015). As a result, the cuticle has a lamellar appearance in cross-section, with each lamella corresponding to the distance in which the orientation of fibers rotates by 180°.

As crustaceans grow and develop, they require an ever-larger exoskeleton. Just as in other arthropods, the crustacean exoskeleton does not grow continuously. Instead, crustaceans periodically secrete a new cuticle and shed the old one, growing, developing and regenerating damaged structures in a series of molts.

Terrestrial isopods or woodlice (Oniscidea) have successfully established themselves in terrestrial environments. Different woodlice are found in a wide range of habitats, including amphibious as well as desert species (Hornung, 2011). As proposed by Schmalfuss (1984), woodlice with similar body types and life-styles can be classified into morphotypes which evidently evolved convergently in several lineages. Some of these morphotypes reflect different strategies of defense against predators. The rollers,

which roll into a ball when threatened, are slow and have thick tergites that protect them from predators. The runners have elongated bodies and long legs and rely on speed to escape predators. The clingers have flat, oval bodies and cling to the substrate when threatened, preventing predators from reaching their vulnerable undersides.

Differences in cuticle thickness, structure and composition have been found between animals with different defense strategies and habitat preferences (Neus *et al.*, 2007; Hild *et al.*, 2008; 2009; Ayari *et al.*, 2016; Wood *et al.*, 2017; Csonka *et al.*, 2018). While cuticle thickness in large adults in a given population is fairly consistent between specimens (Csonka *et al.*, 2018; Khemaissia *et al.*, 2018), little is known about the changes in the thickness and structure of the exoskeletal cuticle during the growth of terrestrial isopods. Furthermore, there is currently no information on how the ultrastructural characteristics of the cuticle, such as the relative thickness of its layers or chitin-protein fiber stacking, change as it becomes thicker.

The best studied species of woodlice are undoubtedly the roller *Armadillidium vulgare* (Latreille, 1804) and the clinger *Porcellio scaber* Latreille, 1804. Among other aspects of their biology, the structure and composition of their cuticles have been studied in more detail than in any other woodlouse (Neus *et al.*, 2007; Hild *et al.*, 2008; Seidl *et al.*, 2012). The tergal cuticle in *A. vulgare* is thicker than in *P. scaber* when adults of similar body lengths are compared, which may reflect their different defense strategies (Hild *et al.*, 2008; Seidl *et al.*, 2012). However, it is not known if the cuticles in these species become thicker or how the structure of their cuticles changes as these animals grow.

In this study, we determined the principles of upscaling of the exoskeletal cuticle in *A. vulgare* and *P. scaber*. We performed a morphometric analysis of cuticles from individuals of different sizes. Our goals were to determine whether the cuticle thickens with growth and how the relative contributions of different cuticular layers change if this occurs. We also aimed to show whether the thickness of cuticular lamellae changes with growth. One possible mechanism by which the cuticle could become thicker would be stacking more layers of chitin-protein fibers with

similar changes in fiber orientation, resulting in more numerous yet similarly thick lamellae in thicker cuticles. Alternatively, sequential planes of chitin-protein fibers might change their orientation by a different angle in thicker cuticles, resulting in a change of lamellar thickness. Our aim was to establish which of these potential mechanisms characterizes changes in cuticles thickness in the studied species.

MATERIAL AND METHODS

Isopod culture

Cultures were established from 2 ovigerous females of *A. vulgare* and 12 adults of *P. scaber* of both sexes. The isopods were sampled in Središče ob Dravi in northeastern Slovenia in July 2016. They were maintained in culture at room temperature ($21 \pm 1^\circ\text{C}$) and a natural light and day rhythm in Ljubljana, Slovenia. The cultures were kept in containers with soil from the sampling site and hazel leaf litter as food. Occasionally, fresh potatoes, zucchini and carrots were added to the culture as additional food items.

The offspring of collected isopods was sampled from the culture at different body sizes over the course of several months. In the case of *A. vulgare*, manca larvae less than 3 days after release from the brood pouch were the youngest specimens measured. Mancae were not obtained from the culture of *P. scaber* and the smallest individuals analyzed in this species were juveniles. Animals that did not possess sternal CaCO_3 deposits that indicate the premolt stage of the molt cycle (Zidar *et al.*, 1998) were fixed in absolute ethanol and used for microscopic analyses. In total, 18 specimens of *A. vulgare* and 25 specimens of *P. scaber* were analyzed.

Imaging

For measurements of body lengths and cephalothorax widths of animals, fixed specimens were imaged in ethanol with an MZ FLIII stereomicroscope equipped with a DFC425 C camera (both from Leica).

For scanning electron microscopy (SEM), tergites from pereonites 2–4 were dissected, further dehydrated with two changes of absolute ethanol and air-dried. Dry tergites were attached to aluminum holders with adhesive carbon disks (SPI supplies),

fractured sagittally to obtain cross-sectional surfaces and sputter-coated with platinum using an SCD 050 sputter coater (Bal-Tec). Images were obtained with a JSM-7500F field emission scanning electron microscope (JEOL).

Measurements and statistical analyses

Body lengths and cephalothorax widths of the woodlice were measured in stereomicroscope images. Cephalothorax width is an established measure for intraspecific comparisons (Sutton, 1968), as it is not influenced by differences in the overlap of sequential body segments. Nevertheless, the shape of the cephalothorax is a species-specific character. To facilitate interspecific comparisons, body length was chosen as a measure of body size in this study. It has been used reliably for both intra- and interspecific comparisons before (Montesanto *et al.*, 2012; Karagkouni *et al.*, 2016).

The thickness of the entire cuticle, the endocuticle and the exocuticle were measured for each individual on SEM images. In the endocuticle, the thickness of lamellae were measured and the measurements averaged to obtain a single value, the average lamellar thickness for that individual. The membranous layer, when present, was not included in measurements concerning the endocuticle. In the exocuticle, the thickness of the distal exocuticle (the distal smooth layer) was measured for each individual. In addition, the thickness of visible lamellae in the proximal exocuticle were determined and averaged to obtain a single average value for each individual. For each parameter, one measurement was performed per individual on images of sagittally fractured tergites in their central regions, approximately halfway along their length. All measurements were performed on pereonites 2–4 using Fiji software (Schindelin *et al.*, 2012).

Coefficients of correlation and the corresponding significance values were determined for selected pairs of morphometric parameters. Differences between species were evaluated with ANCOVA with species as independent variables, morphometric features as dependent variables and cuticle thickness as the covariate. These analyses were performed in Past3 (Hammer *et al.*, 2001). To compare the relationship

between body length and cuticle thickness, the difference of regression slopes between species was tested with the test of regression slope differences for independent samples (Blejec, 1973).

RESULTS

Overview of cuticle structure

Sagittally fractured anterior tergites of *A. vulgare* and *P. scaber* (Fig. 1) are presented in Figs. 2 and 3, respectively. The cuticle in both species consists of a thin epicuticle, an exocuticle and an endocuticle, with a membranous layer of variable thickness which may also be absent. The distal smooth layer, corresponding to the distal exocuticle, can be observed just beneath the epicuticle (Figs. 2, 3). Regardless of the size of the individuals, the lamellar appearances

of the endocuticle and the proximal exocuticle are discernible. It is evident that larger animals have thicker cuticles with thicker endocuticular lamellae.

Relationship between cephalothorax width and body length

In *A. vulgare* and *P. scaber*, cephalothorax width is strongly correlated with body length ($r = 0.98$ and $p < 10^{-12}$ for both species; Fig. 4). This is a good indication that body length is an appropriate measure of body size for both intra- and interspecific comparisons. Specimens of *P. scaber* grew to a body length of 12.5 mm in culture. In the case of *A. vulgare*, individuals grew to a maximum size of 11 mm during the study. The animals grown in culture thus reached body sizes characteristic of the species and similar to the animals that were obtained in nature and founded the culture.

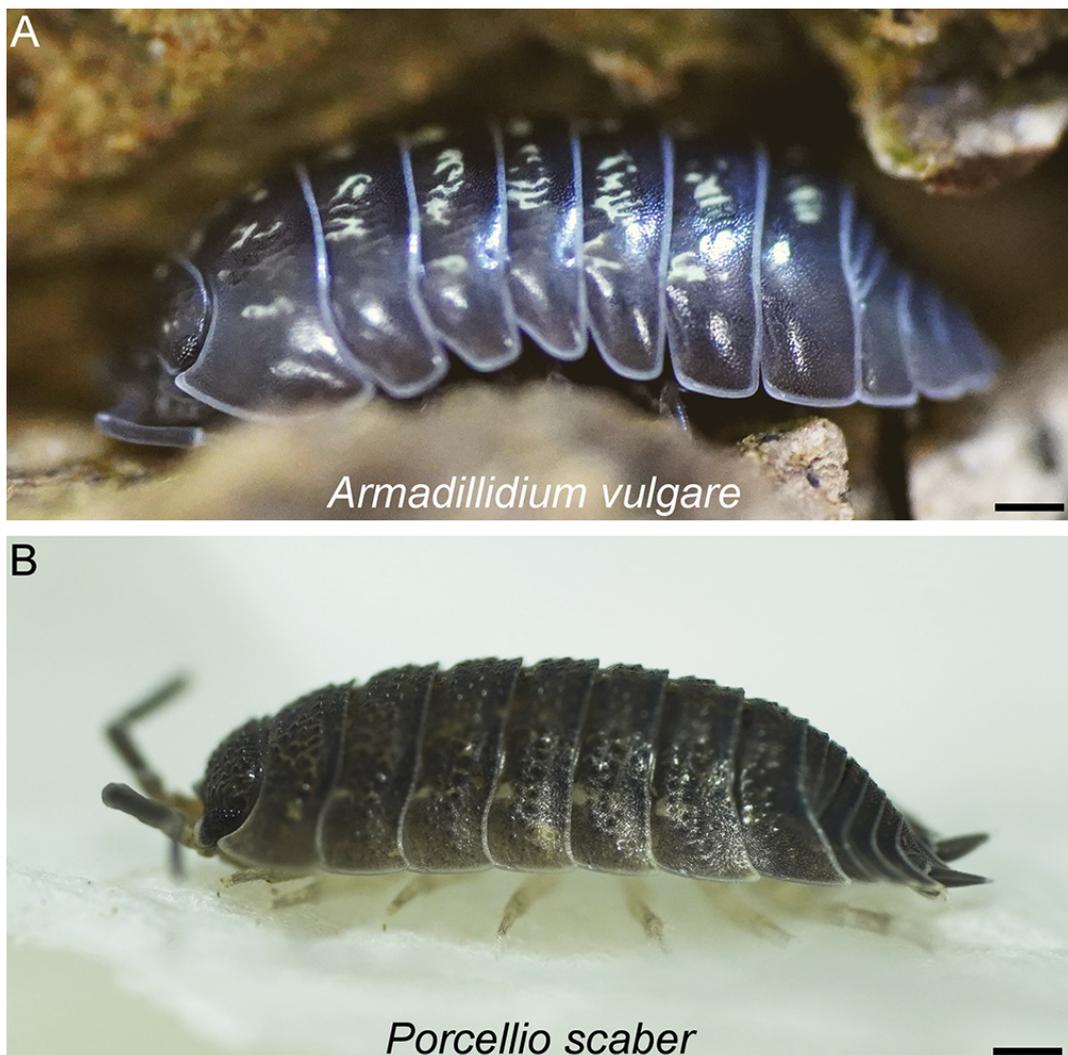


Figure 1. Studied species. **A**, *Armadillidium vulgare*. **B**, *Porcellio scaber*. Scale bars = 1 mm. Photographs by Ana Sterle.

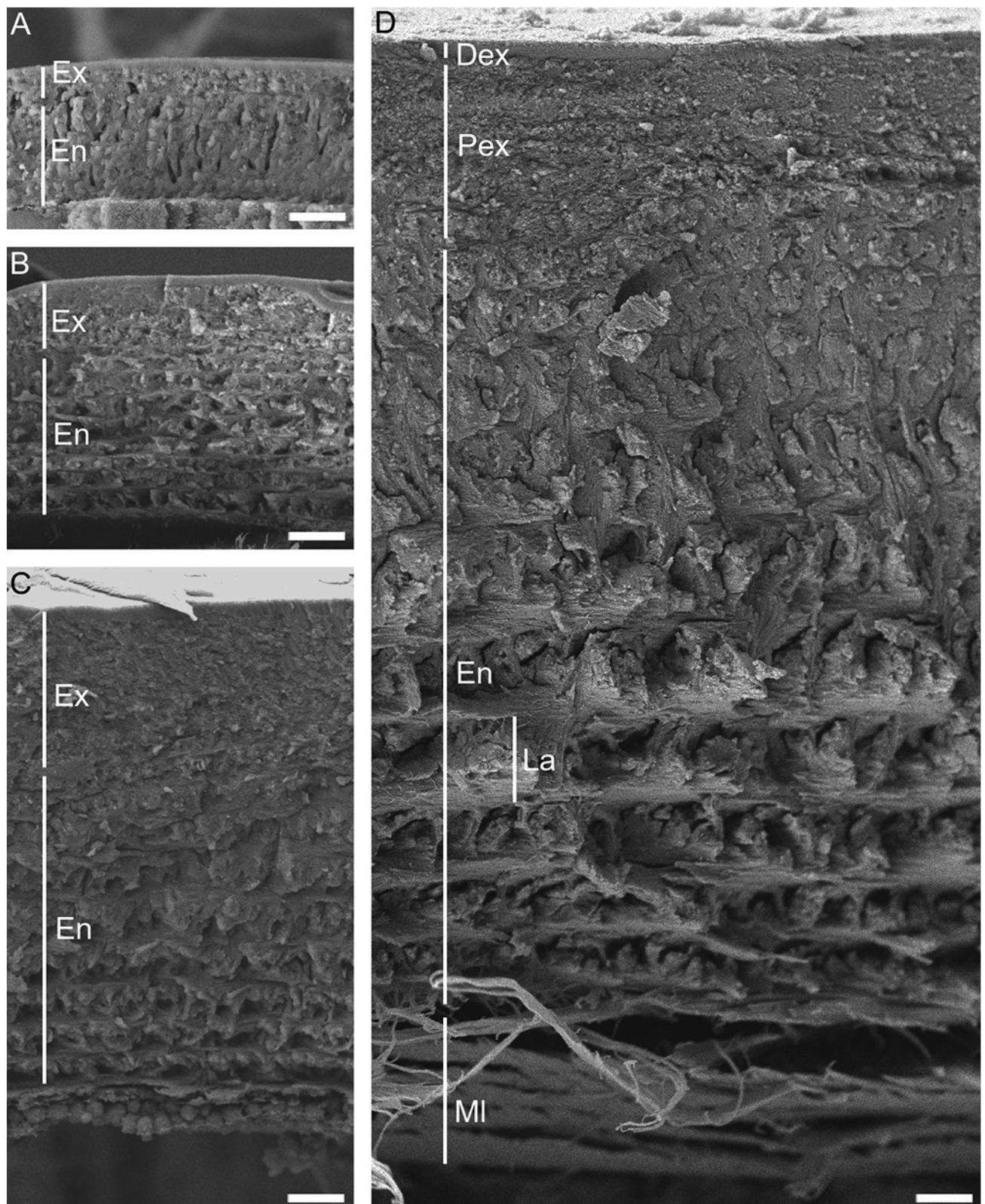


Figure 2. Scanning electron micrographs of sagittally fractured anterior tergites of the pereon from *Armadillidium vulgare* individuals of different sizes, shown to scale. **A**, Manca larva; 1.5 mm body length. **B**, 2.2 mm body length. **C**: 4.5 mm body length. **D**, 11 mm body length. Scale bars = 2 μ m. Dex: distal exocuticle; En: endocuticle; Ex: exocuticle; La: lamella in the endocuticle; MI: membranous layer; Pex: proximal exocuticle.

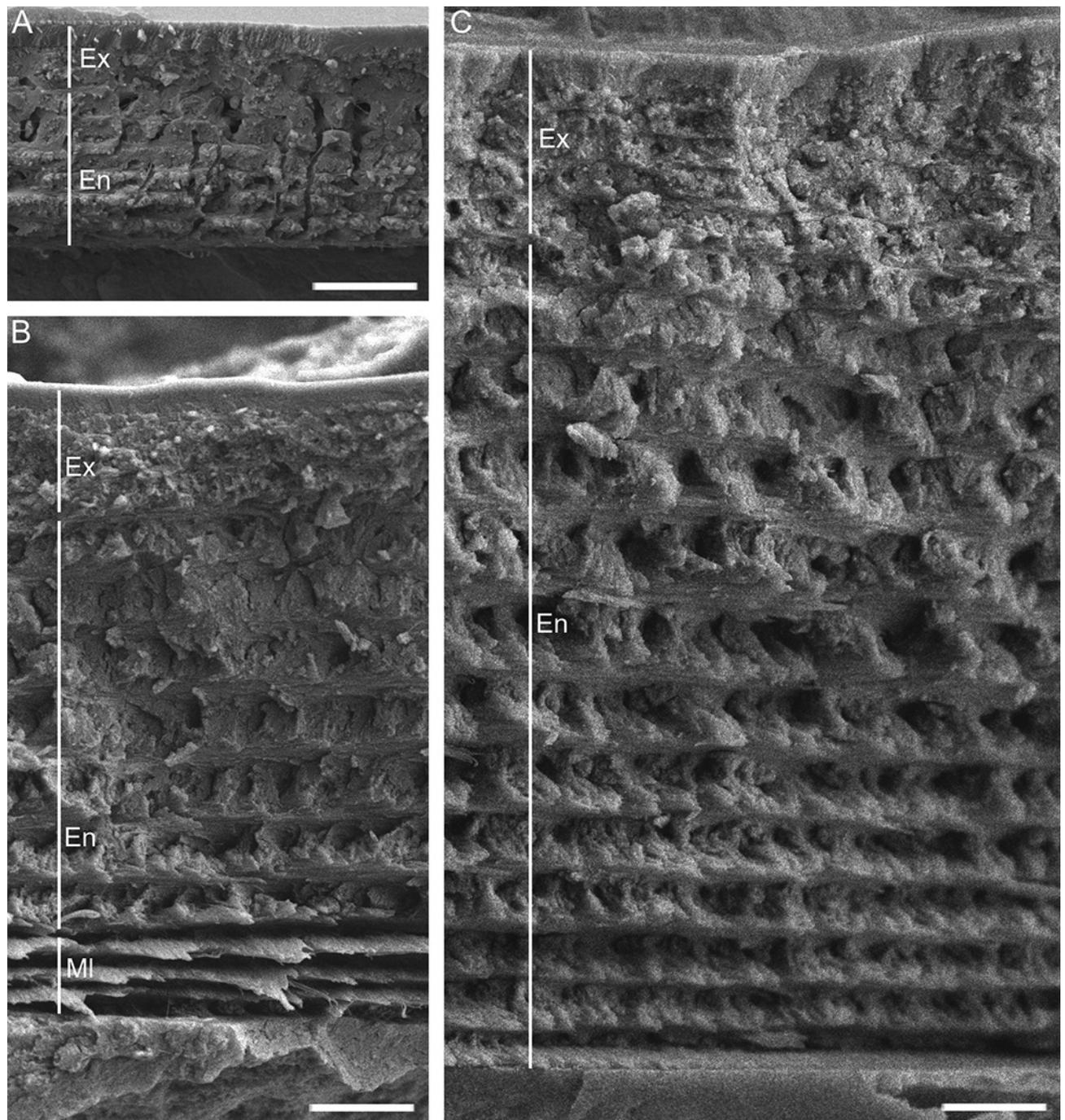


Figure 3. Scanning electron micrographs of sagittally fractured anterior tergites of the pereon from *Porcellio scaber* individuals of different sizes, shown to scale. **A**, 4.2 mm body length. **B**, 7 mm body length. **C**, 10 mm body length. Scale bars = 2 µm. En: endocuticle; Ex: exocuticle; MI: membranous layer.

Tergal cuticle thickness in relation to body size

In both species, the thickness of the tergal cuticle correlates with body length ($r = 0.82$ for *A. vulgare* ($p = 6.7 \times 10^{-5}$) and 0.67 for *P. scaber* ($p = 2 \times 10^{-4}$) (Fig. 5). The slope of the relationship between body length and cuticle thickness is greater for *A. vulgare* ($p = 0.002$).

The thickness of tergites therefore increases with increasing body length at a higher rate in *A. vulgare* than in *P. scaber*. Even in animals grown in culture and thus under similar conditions, the variability in cuticle thickness between conspecifics of similar body sizes is fairly large.

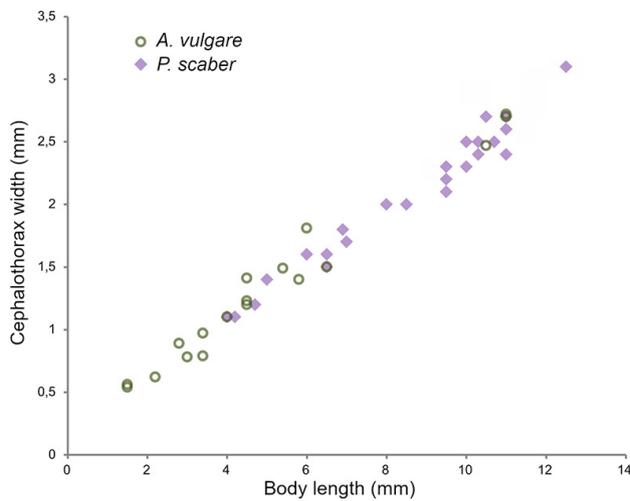


Figure 4. Relationship between body length and cephalothorax width in *Armadillidium vulgare* (circles) and *Porcellio scaber* (diamonds).

In *A. vulgare*, the thickness of the tergal cuticles ranged between $3.5\ \mu\text{m}$ (in a 1.4 mm long manca larva less than 3 days after release) and $39.5\ \mu\text{m}$ (in an 11 mm long individual; Fig. 2). In *P. scaber*, the thickness ranged between $4.4\ \mu\text{m}$ (in a 4.2 mm long juvenile) and $21.2\ \mu\text{m}$ (in a 10 mm long specimen; Fig. 3).

Changes in cuticle structure during growth

As the cuticle grows, the endocuticle thickness to cuticle thickness ratio increases ($r = 0.59$, $p = 0.001$ for *P. scaber* and $r = 0.90$, $p = 8 \times 10^{-7}$ for *A. vulgare*; Fig. 6A). Thus, the relative contribution of the endocuticle in the tergal cuticle becomes larger in both species as they grow. As determined by ANCOVA, the percentage of cuticle thickness represented by the endocuticle does not differ significantly between the two species if adjusted for cuticle thickness as a covariate.

There is a strong correlation between the average thickness of the endocuticular lamellae and the thickness of the cuticle in both species ($r = 0.86$ for *A. vulgare* and $r = 0.88$ for *P. scaber*; $p < 10^{-5}$ for both species; Fig. 6B). The average thickness of endocuticular lamellae ranged between 360 nm and 2880 nm in *A. vulgare* and between 430 nm and 1300 nm in *P. scaber*. The lamellae were thickest in the thickest cuticles — in large specimens of *A. vulgare*. There was no demonstrable difference in the thickness of the endocuticular lamellae between species when controlling for cuticle thickness as a covariate.

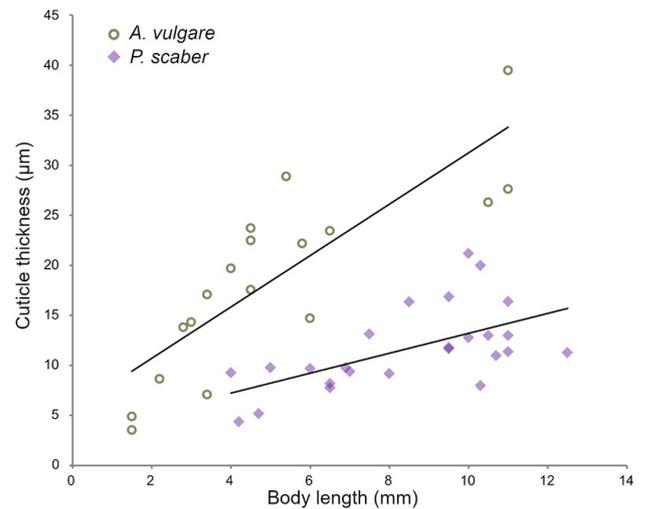


Figure 5. Relationship between the body length and the tergal cuticle thickness in *Armadillidium vulgare* (circles; $r = 0.82$) and *Porcellio scaber* (diamonds; $r = 0.67$) with corresponding regression lines.

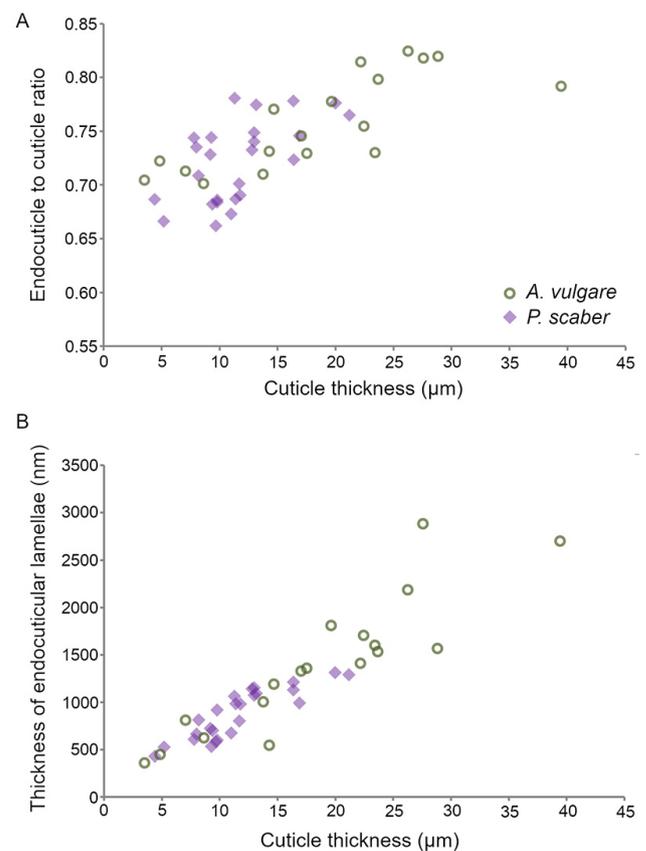


Figure 6. Changes in cuticle structure during growth. **A**, Relationship between the cuticle thickness and the ratio between the thickness of the endocuticle and the thickness of the cuticle in *Armadillidium vulgare* (circles) and *Porcellio scaber* (diamonds). **B**, Relationship between the cuticle thickness and the average thickness of the endocuticular lamellae in *A. vulgare* (circles) and *P. scaber* (diamonds).

While the correlation between the cuticle thickness and the thickness of the visible lamellae in the proximal exocuticle is significant in *A. vulgare* ($r = 0.62$, $p = 0.006$; Fig. 7A), there is no convincing correlation between these parameters in *P. scaber* ($r = 0.40$, $p = 0.05$). There is also no demonstrable correlation between the cuticle thickness and the thickness of the distal exocuticle in either of the two species ($r = 0.56$, $p = 0.02$ and $r = 0.11$, $p = 0.61$ for *A. vulgare* and *P. scaber*, respectively; Fig. 7B). Once again, no significant differences in these characteristics were demonstrated between *A. vulgare* and *P. scaber*.

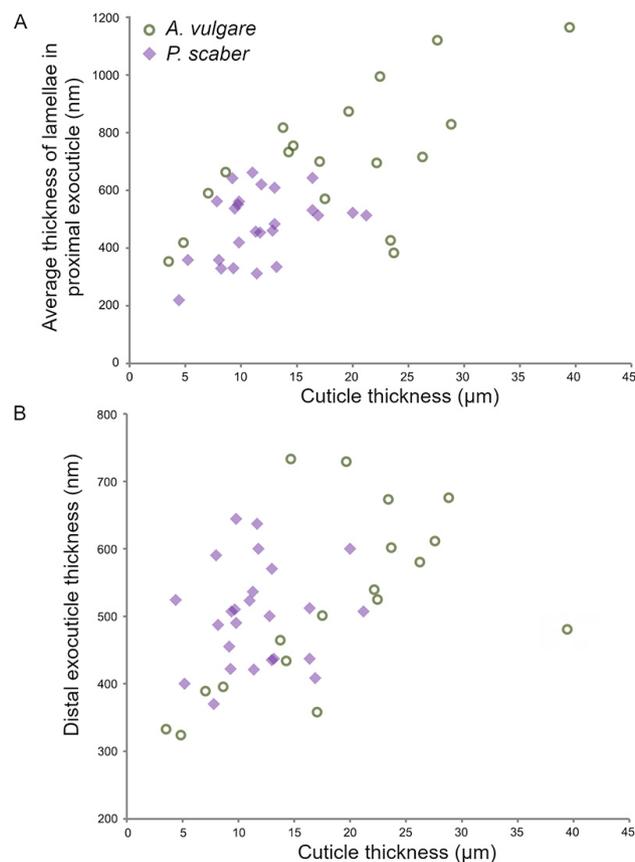


Figure 7. Changes in the exocuticle during growth. **A**, Relationship between the cuticle thickness and the average thickness of the lamellae in the proximal exocuticle in *Armadillidium vulgare* (circles) and *Porcellio scaber* (diamonds). **B**, Relationship between the cuticle thickness and the thickness of the distal exocuticle (smooth layer) in *A. vulgare* (circles) and *P. scaber* (diamonds).

DISCUSSION

As woodlice grow, their tergal cuticles grow thicker with successive molts. Among terrestrial isopods, larger individuals have been demonstrated to have thicker tergal cuticles in two species of trichoniscids as

well (Vittori and Štrus, 2014). In *P. scaber*, comparisons have been made between the cuticles of embryos, marsupial mancae – the larval stages of peracarids not yet released from the brood pouch – and adults (Mrak *et al.*, 2014). The conclusions were that the cuticle of marsupial mancae is structurally similar to adult cuticles and is already mineralized, but is much thinner. A similar trend of cuticle thickening with increasing body size has previously been demonstrated in benthic marine malacostracans, including a benthic isopod, whereas cuticle thickness was shown to remain constant during growth in pelagic species (Pütz and Buchholz, 1991; Waugh *et al.*, 2009). Changes in the thickness of the integument during ontogenetic development may have a profound impact on woodlice biology. If the thickness of the cuticle determines desiccation resistance, which is likely the case (Csonka *et al.*, 2018), a thinner exoskeletal cuticle in smaller individuals would make them more vulnerable to water loss and could influence their microhabitat preferences.

The cuticle of the roller *A. vulgare* is not only thicker at comparable body sizes; its thickness also increases at a higher rate than in *P. scaber*. The rate of cuticle thickening may be linked to the isopod's defense strategy. As a roller, *A. vulgare* potentially requires a greater increase of cuticle thickness at a given increase of body size if the cuticle is to perform its defensive function in larger animals.

With increasing cuticle thickness, the proportions of different layers in the cuticle change considerably. The relative contribution of the endocuticle increases, whereas the exocuticle represents an ever-smaller share of the cuticle. This is even more pronounced in the distal exocuticle, which hardly thickens at all. A similar trend has been recognized in the carapace cuticle of the blue crab, *Callinectes sapidus* Rathbun, 1896, in which the proportion of the exocuticle in the carapace cuticle diminishes as the cuticle becomes thicker (Waugh *et al.*, 2009).

There are many possible consequences of these changes in layer proportions. In *A. vulgare* as well as in *P. scaber*, the exocuticle is mineralized predominantly with calcite, with amorphous calcium carbonate (ACC) present predominantly in pore canals, whereas the endocuticle is mineralized with ACC and calcium phosphate (Hild *et al.*, 2008; Seidl *et al.*,

2012). Mineralization of the endocuticle with ACC may facilitate the resorption and potential reuse of mineral from the exoskeleton during molt, as the highly soluble ACC is predominantly resorbed from the cuticle (Neues *et al.*, 2011). Temporary deposition and reutilization of minerals are possible in terrestrial isopods as ACC from the endocuticle may be deposited in sternal CaCO₃ deposits during preparation for molt (Ziegler *et al.*, 2005). A relatively thicker endocuticle thus potentially enables the recycling of a greater percentage of the mineral in the exoskeleton. The resorption and storage of minerals may be of greater importance in thick cuticles which require large amounts of calcium, phosphorus and carbonate for their mineralization (Seidl *et al.*, 2012). This has previously been suggested as an explanation of the differences in composition between the cuticles of rollers and clingers, but our results show that it may be extended to differences between thicker and thinner cuticles in individuals of the same species.

A second likely consequence of the changes in relative contributions of different cuticular layers is the altering of the mechanical properties of the tergites. As the calcite-containing layers are harder and more brittle than the layers mineralized with ACC (Seidl *et al.*, 2012), the positioning of the calcite-containing exocuticle over the more pliable and fracture-resistant endocuticle likely increases the strength of the tergites, as the endocuticle is subjected to greater tension upon loading (Seidl *et al.*, 2012). This idea has not been extended to consider the effects of the relative layer thickness on the mechanical properties of the tergite as a whole, but we can expect a larger part of thicker cuticles to be softer.

At this point, we naturally cannot be certain that any of these potential adaptive advantages actually resulted in selection for the observed changes in proportions of cuticular layers with growth. It is just as possible that the exocuticle, which is deposited before molt, does not thicken much during growth due to the physiological mechanisms underlying cuticle deposition and the need for its expansion after the shedding of the old cuticle.

With the growth of the cuticle, endocuticular (and, at least in *A. vulgare*, exocuticular) lamellae become thicker. The lamellae of crustacean endocuticles can be interpreted as stacks of planes in which chitin-

protein fibers periodically shift their orientation in accordance with the Bouligand pattern (Seidl *et al.*, 2011). Thus, thicker lamellae result from fibers shifting their orientation more gradually, with sequential layers of fibers changing direction by a smaller angle (Vittori and Štrus, 2014). Although the mechanical effects of changing lamellar thickness in cuticles with similar composition remain to be experimentally determined, existing studies indicate that thinner lamellae result in harder cuticles. Cuticular lamellae have been shown to be thicker in benthic than in pelagic malacostracans and this has been interpreted in view of the mechanical properties of the cuticle. The proposed explanation was that thinner lamellae provide more rigidity to the relatively thinner cuticles in pelagic crustaceans (Pütz and Buchholz, 1991). Without considering possible differences in mineral content, layers of the cuticle consisting of thinner lamellae have also been shown to be harder in a decapod (Raabe *et al.*, 2005).

Overall, we can theoretically expect thinner lamellae and a relatively thicker calcite-containing exocuticle to result in an increased hardness of thinner cuticles. Thicker cuticles, by contrast, should overall be less hard with more calcium carbonate and calcium phosphate available for mobilization during molt.

Despite the fact that *A. vulgare* and *P. scaber* are species with different defense strategies, one being a roller and the other a clinger, there are no morphometric differences in the ultrastructure of their cuticles if the effects of cuticle thickness are taken into consideration. While the tergal cuticle is consistently thicker in *A. vulgare*, it is apparently structured precisely as the cuticle of *P. scaber* would be if it were similarly thick. Previous studies have showed that the cuticle of *A. vulgare* contains relatively more mineral than the cuticle of *P. scaber*. Also, more mineral is in the form of amorphous calcium carbonate and there is less calcite (Neues *et al.*, 2007). As the cuticle is thicker in *A. vulgare*, these differences in mineral composition can be explained as resulting from a consequently smaller contribution of the exocuticle, without an inherent difference in composition between the cuticles of rollers and clingers.

Nevertheless, it is known that cuticles of similar thickness are structured differently in certain species when compared to *A. vulgare* and *P. scaber*. Ayari *et al.* (2016) reported that the relative thickness of

the exocuticle in the tergites of the desert isopod *Hemilepistus reaumurii* (Milne-Edwards, 1840) is greater than that determined in *A. vulgare* and *P. scaber* in our study. A species that is morphologically transitional between runners and clingers, *Balloniscus glaber* Araujo and Zardo, 1996, has much thinner endocuticular lamellae than those measured in *A. vulgare* and *P. scaber* (Wood et al., 2017). Troglobitic species from European caves similarly have very numerous and thin endocuticular lamellae in addition to very thin cuticles for their body sizes (Vittori and Štrus, 2014). Furthermore, the pattern of calcite and ACC distribution characteristic of *A. vulgare* and *P. scaber* tergites is not universal; only roughly the external half of the exocuticle is mineralized with calcite in the cavernicolous woodlouse *Titanethes albus* (C. Koch, 1841) with the rest of the cuticle containing ACC (Hild et al., 2009). In the beach isopod *Tylos europaeus* Arcangeli, 1938, only the distal exocuticle contains calcite (Seidl et al., 2011).

The main conclusion we can draw is that the thickness and the structure of the tergal cuticle change continuously during the growth and development of terrestrial isopods. This likely affects traits such as water loss and the ability to evade predators during different life-cycle stages. The structural and compositional differences between exoskeletons of different terrestrial isopods are likely the result of an interplay of phylogeny, defense strategies and adaptations to different habitats, with animal size being an important factor as well.

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