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Development and ultrastructure of the rigid dorsal and flexible ventral cuticles of the elytron of the red flour beetle, *Tribolium castaneum*

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ABSTRACT

Insect exoskeletons are composed of the cuticle, a biomaterial primarily formed from the linear and relatively rigid polysaccharide, chitin, and structural proteins. This extracellular material serves both as a skin and skeleton, protecting insects from environmental stresses and mechanical damage. Despite its rather limited compositional palette, cuticles in different anatomical regions or developmental stages exhibit remarkably diverse physicochemical and mechanical properties because of differences in chemical composition, molecular interactions and morphological architecture of the various layers and sublayers throughout the cuticle including the envelope, epicuticle and procuticle (exocuticle and endocuticle). Even though the ultrastructure of the arthropod cuticle has been studied rather extensively, its temporal developmental pattern, in particular, the synchronous development of the functional layers in different cuticles during a molt, is not well understood. The beetle elytron, which is a highly modified and sclerotized forewing, offers excellent advantages for such a study because it can be easily isolated at precise time points during development. In this study, we describe the morphogenesis of the dorsal and ventral cuticles of the elytron of the red flour beetle, *Tribolium castaneum*, during the period from the 0 d-old pupa to the 9 d-old adult. The deposition of exocuticle and mesocuticle is substantially different in the two cuticles. The dorsal cuticle is four-fold thicker than the ventral. Unlike the ventral cuticle, the dorsal contains a thicker exocuticle consisting of a large number of horizontal laminae and vertical pore canals with pore canal fibers and rib-like veins and bristles as well as a mesocuticle, lying right above the enodcuticle. The degree of sclerotization appears to be much greater in the dorsal cuticle. All of these differences result in a relatively thick and tanned rigid dorsal cuticle and a much thinner and less pigmented membrane-like ventral cuticle.

1. Introduction

Molting of the exoskeleton or cuticle is an essential developmental process for many invertebrate species in which the extracellular matrix or cuticle is replaced and remodeled during growth. The insect cuticle is produced by the underlying epidermal cells of ectodermal origin. It provides protection against mechanical injury, pathogens and parasites, while also providing sensory perception, muscle attachment points, coloration, camouflage and other physiological functions. Since the cuticle is present at all developmental stages and essentially covers the entire body, it must exhibit great diversity in its physicochemical and mechanical properties to allow for growth and to accommodate the functions of the tissues and organs that are protected. Thus cuticles from different parts of the insect's anatomy or the same cuticle present at different developmental stages may have widely different mechanical properties that arise most likely from differences in chemical composition and molecular interactions, as well as the thickness and arrangement of morphologically distinct layers within the cuticle.

Insect cuticle is composed primarily of chitin, which is a rather rigid polysaccharide that accounts for much of the mechanical strength of the cuticle, and a whole assortment of proteins, some structural many of which bind to chitin and others are enzymes that catalyze chitin deacetylation, tanning or cross-linking of structural proteins to one another. The ultrastructure of the cuticle has been studied extensively by optical microscopy, scanning

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electron microscopy, transmission electron microscopy and X-ray tomography in a variety of insect species, most notably in the Coleoptera (Arwin et al., 2013; Banerjee, 1988; Dai and Yang, 2010; del Rio et al., 2014; Lenau and Barfoed, 2008; Leopold et al., 1992; Locke and Huie, 1979; Moussian et al., 2006; Roux-Pertus et al., 2017; Sobala and Adler, 2016; van de Kamp et al., 2016). Generally, the cuticle has been divided into several morphologically and functionally distinct layers (Locke, 2001). The outermost layer is the envelope, which may contain several sublayers. It primarily consists of waxes and other lipids and occasionally even carbohydrates, and provides a water-proofing function. Below the envelope is the epicuticle, which is more electron dense and contains phenolics used for tanning and proteins. The rest of the cuticle is broadly termed the procuticle and has been further subdivided into the exocuticle and endocuticle. The rigid adult Coleopteran cuticle may also include a middle transitional layer, the mesocuticle (Cheng et al., 2009; Noh et al., 2016b). In general, the term exocuticle and endocuticle are associated with layers formed before and after molting, respectively. In most cases the exocuticle is tanned and consists of helicoidally arranged laminae with alternating light and dark regions when viewed under a transmission electron microscope. At least in some beetle species, the endocuticle has a distinctly different brick-like structure, denoted as “Balken cuticle” or “macrofibers” (Cheng et al., 2009; Leopold et al., 1992; Noh et al., 2016b; Roux-Pertus et al., 2017; van de Kamp et al., 2016). Some cuticles also have vertically oriented pore canals that appear to originate from the apical plasma membrane of epidermal cells and terminate in the epicuticle (Noh et al., 2014, 2015).

Even though a great deal of information is available regarding the structure of arthropod cuticle, there have been only a few studies on the developmental pattern of growth of the cuticle and its constituent layers and sublayers. The variations of the properties of cuticular layers, such as thick vs. thin, helicoidal vs. pseudo-orthogonal, tanned vs. untanned, laminate vs. non-laminate, imply that a complexity exists in the way these layers are deposited during development. This has, in fact, been shown to be the case in a limited number of insect species (Leopold et al., 1992; Locke and Huie, 1979). During formation of a new cuticle, there is a general sequence of appearance of the envelope, the epicuticle and the procuticle (exocuticle and endocuticle) in that order in different species. In many cases, each layer is completed before the appearance of the underlying layer. For example, those three layers in the wing cuticle of the fruit fly, *Drosophila melanogaster*, are formed according to a hierarchical order in which the genes expressed in a given layer (for example, envelope, epicuticle) appear to guide the deposition of the layers that lie immediately below them (Sobala and Adler, 2016). However, in the embryonic cuticle of this species, simultaneous growth of the epicuticle and procuticle has been reported (Moussian et al., 2006). Whether there is a synchronous development of these layers in cuticles of other species during a molt has not been determined.

The beetle elytron, which is a highly modified, thickened and sclerotized forewing that protects the more delicate hindwing (Tomoyasu et al., 2009), offers excellent advantages for such a study of cuticle formation because it can be easily isolated at precise time points during development and can be analyzed using a variety of physical, microscopic and biochemical techniques. It has the added advantage of having two types of cuticle, both a relatively thick rigid dorsal cuticle and a thin flexible ventral cuticle separated by hemolymph space and many trabeculae that represent supporting braces between these two layers (Arakane et al., 2012; Chen and Wu, 2013; Ni et al., 2001; Noh et al., 2016b). Some of the mechanical properties and three-dimensional structural characteristics of the elytron have been elucidated (Chen et al., 2015a, 2015b; Lomakin et al., 2010, 2011; Zhang et al., 2017). In addition, the proteomics and transcriptomics of the elytron of the red flour beetle, *Tribolium castaneum*, have been previously described (Dittmer et al., 2012). In this report, we describe the temporal deposition of different layers of the rigid dorsal and flexible ventral cuticles of the elytron of *T. castaneum*, as well as the maturation of pore canals that help to stabilize the dorsal cuticle.

2. Material and methods

2.1. Insects

The *T. castaneum* strain GA-1 was used to study adult cuticle development. Insects were reared at 30 °C at 50% relative humidity in whole wheat flour fortified with 5% (v/v) Brewer's yeast. Under this rearing condition, adult eclosion occurs 5 days after pupation.

2.2. Scanning electron microscopy (SEM)

Elytra were dissected from 5 to 7 d-old adults to analyze morphology of the dorsal and ventral cuticles by SEM. The elytron was flash-frozen in liquid nitrogen and then fractured for SEM. Samples were coated with a gold/palladium mixture and viewed using the S-3500N scanning electron microscope (Hitachi).

2.3. Transmission electron microscopy (TEM)

Elytral tissue samples were prepared at different times during pupal and adult development to analyze cuticle ultrastructure as described in a previous report (Noh et al., 2014). Samples were collected from 0 d-old pupae to 9 d-old adults and fixed in 0.1% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 24 h at room temperature. Samples were rinsed three times for 15 min with 0.1 M sodium cacodylate buffer, and then dehydrated in an ethanol gradient of 50, 60, 70, 80, 90, 95 and 100% for 20 min each. The tissues were infiltrated in LR white resin (Electron Microscopy Sciences, PA, USA) in 2:1 ethanol:resin for 4 h, 1:1 ethanol:resin for 8–10 h, 1:2 ethanol:resin for 4 h and 100% resin overnight. Tissues were vacuum-infiltrated for 2 h, placed in gelatin capsules (Electron Microscopy Sciences), and then polymerized at 55 °C for 12–16 h followed by ultrathin sectioning. Ultrathin sections (~90 nm) were stained with 4% aqueous uranyl acetate for 10 min and then imaged using the JEM-1400 transmission electron microscope (JEOL). To detect chitin, ultrathin-sectioned elytra (~90 nm) of mature adults (9-10 d-old adults) were incubated with 10 nm gold-conjugated wheat germ agglutinin (WGA, EY Laboratories) (1:100) in 0.05 M TBS, pH 8 containing 0.05% fish gelatin (BB International) for 2 h at room temperature. The sections were washed with 0.05 M TBS five times for 5 min each, deionized water three times for 5 min each at room temperature, followed by staining with 4% aqueous uranyl acetate for 10 min.

3. Results

3.1. The beetle elytron as a model for cuticle development

The forewing of the Coleoptera called the “elytron” is a highly modified and sclerotized structure that protects beetles from several environmental stresses such as physical damage to the underlying membranous hindwing and dorsal abdomen, predation, desiccation/dehydration and cold shock (Linz et al., 2016). The mature elytron is composed of dual layers of epidermal cells that secrete both a thick dorsal and a thin ventral cuticular lamination (Fig. 1). The former is highly tanned and exhibits bulging veins and

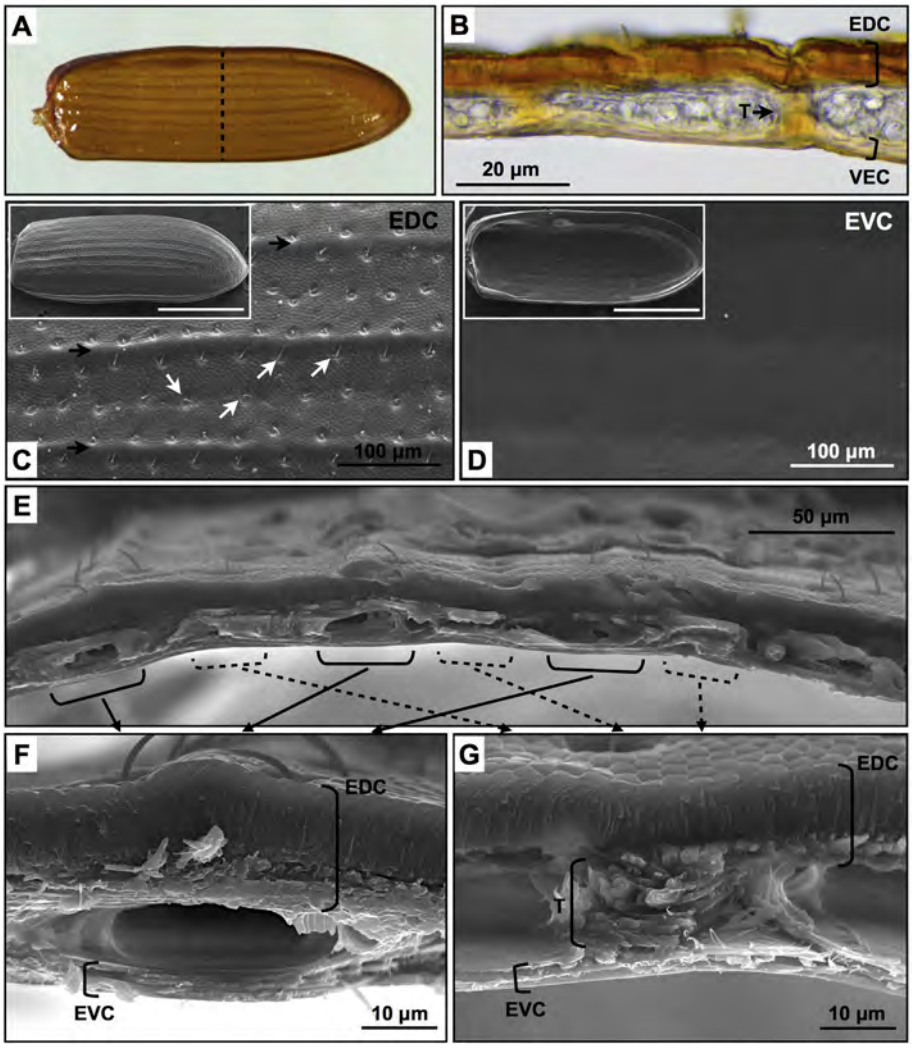

Fig. 1. Light and scanning electron microscopic analyses of the *T. castaneum* elytron. The elytron was collected from a 5-7 d-old adult (A), and embedded in optimal sectioning temperature compound. Cryosections (~16 μm) (broken line in A) were washed with PBS to remove the freezing compound and then observed under an optical light microscope (B). (C and D) The elytral dorsal (EDC) and ventral (EVC) cuticles' surfaces were analyzed by SEM. White and black arrows in C indicate veins and bristles of EDC cuticle, respectively. Scale bar in insets is 2 mm. (E–G) The elytron was also flash-frozen in liquid nitrogen, fractured and prepared for SEM. Multiple layers were observed in EDC and EVC cuticles of the elytron. Note a cavernous fortifying structure (solid brackets and arrows) between the EDC and EVC cuticles due to development of supporting beam-like structures (broken brackets and arrows) known as trabeculae (T).

many bristles (Fig. 1C). Its ultrastructure is much like that of other pigmented rigid cuticles in different body regions of adult beetles such as the leg and ventral abdomen (Noh et al., 2015). In contrast, the ventral cuticle is less pigmented, smoother, thinner and membranous (Fig. 1D) like the hindwing and the dorsal abdominal cuticle. Both layers are proximate to each other immediately after adult eclosion. In the mature adult, however, there is a substantial space between these two layers, which is filled with hemolymph (Fig. 1B, E and F). This space is interrupted by numerous supporting pillar-like fibrous structures called “trabeculae” (Fig. 1B, E and G), which are cuticular extensions that connect the dorsal and ventral cuticles, and generally have a mechanical function of separating and supporting the dorsal and ventral cuticular layers of the elytron in the vertical direction (Noh et al., 2016a, 2016b). In addition, the elytral dorsal cuticle, but not the ventral cuticle, contains numerous pore canals that start at the apical plasma membrane and end in the epicuticle. In the following sections, we document the development of each of these layers and structures during the period from the 0 d-old pupa to the 9 day-old adult.

3.2. Apolysis of the pupal cuticle

Under our insect rearing conditions, the pupal cuticle remains juxtaposed next to the dorsal epidermal cells 2 h after pupation (2 HAP, Fig. S1A). It then becomes thicker consisting of many horizontal laminae and separates from the epidermal cells (apolysis) by 24 HAP (Fig. S1B). At 54 HAP, the epidermal cells are columnar and elongated (Fig. S1C). The apical plasma membrane is irregular in shape with electron-dense material at the tips of microvilli (arrows in Fig. S1D). Presumably, these are the plasma membrane plaques (PMPs) described by Locke and Huie (1979). There is no evidence of any adult cuticular layer at this stage.

3.3. Envelope and epicuticle formation in the elytral dorsal cuticle

At 60 HAP, microvilli with PMPs become more evenly distributed on the apical plasma membrane (Fig. 2A and B). Just above these PMPs, precursor envelope fragments of the tripartite envelope layer accumulate, but they are disconnected (solid arrow

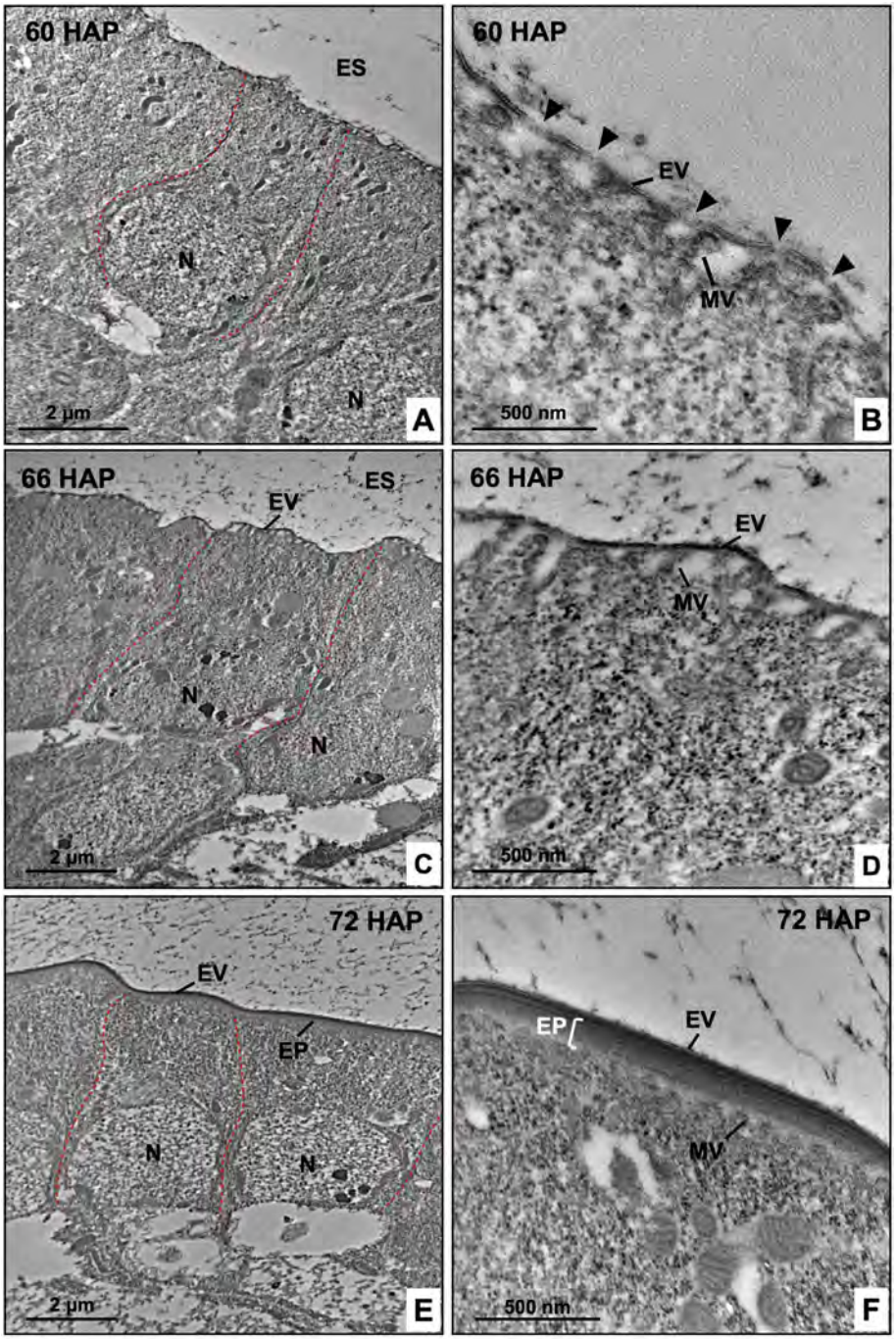

Fig. 2. Epicuticle and exocuticle formation. (A and B) The epidermal cells are columnar at 60 HAP. The apical plasma membrane of the epidermal cells has a serrated appearance with numerous microvilli. The discontinuous envelope (solid arrow heads in B indicate small gaps) is formed at the tip of the apical plasma membrane. (C and D) Epidermal cells become cuboidal at 66 HAP. A large nucleus is localized at the basal side of the epidermal cells. The previously fragmented envelope is now fused to form a continuous layer, which covers the top of the entire epidermis. (E and F) The envelope completely covers the epidermal cells at 72 HAP. The epicuticle is being formed underneath the envelope. ES, ecdysial space; N, nucleus; EV, envelope; MV, microvilli; EP, epicuticle.

heads in Fig. 2B). By 66 HAP, the precursor fragments have fused to form a continuous outer envelope layer above the epidermal cells, establishing the outer border of the newly forming dorsal cuticle (Fig. 2C and D). The epidermal cells become cuboidal and compressed with a large nucleus located near the basal side of the epidermal cells (Fig. 2C).

By 72 HAP, the epidermal cells remain cuboidal and the apical plasma membrane appears to have become smoother following the contours of the envelope layer (Fig. 2E and F). At this time the electron-dense PMPs are no longer prominent and the epicuticle (relatively electron-lucent at this stage) begins to form underneath the continuous envelope layer (Fig. 2E and F).

3.4. Elytral dorsal exocuticle formation between 78 and 90 HAP

By 78–84 HAP, the epidermal cells have become broader (Fig. 3A and C), and the electron-dense tips of the microvilli on the wavy apical plasma membrane are evident as seen at 72 HAP even

though they seem to be flattened as well (Fig. 3B and D). There are several alternating dark and light lamellae in the epicuticle, which become more electron-dense compared to that observed at the previous stage (Fig. 3B and D). The exocuticle that is forming underneath the epicuticle consists of several alternating electron-dense and electron-lucent laminae (Fig. 3B and D). There are many pore canals that cross the horizontal laminae in a vertical direction (normal to the epidermal cell surface), which appear to arise directly from the protrusions of apical plasma membrane of the underlying epidermal cells into the procuticle. These apical plasma membrane protrusions (APMPs) may be cytoplasmic extensions with plasma membrane boundaries that penetrate the horizontal laminae and reach all the way to the epicuticle presumably to deliver components for assembly of cuticular layers (Fig. 3B and D).

At 90 HAP, the apical plasma membrane exhibits prominent and evenly distributed microvilli (Fig. 3E and F). The pore canals are more numerous and broader and are filled with unorganized fibers (pore canal fibers, PCFs) that extend from regions at the top of the APMPs, nearly to the bottom of the epicuticular layer (Fig. 3E and F). The assembly zone containing amorphous fibrous materials (chitin) is observed at the interface between the epidermal cells right above the microvilli (Fig. 3F and G), presumably serving as assembly sites for the forming exocuticular laminae containing both chitin and proteins. The cross-section of the middle of exocuticle reveals the circular/oval pore canals containing electron-dense materials in their cores (Fig. 3H).

3.5. Maturation of elytral dorsal cuticle from pharate adult to adult stages

By 120 HAP, the last day of the pupal/pharate adult stage, the exocuticle has thickened dramatically and rapidly to a size of approximately ~6–7 μm (Fig. 4A). There are numerous compact laminae of alternating electron-dense and -lucent layers in the exocuticle (Fig. 4A). Some of the pore canals are branched near the epicuticle (Fig. 4B). Pore canals at this stage are clearly more organized when compared to earlier stages and consist of an electron-lucent space in the middle surrounded by a circular column made of thick PCFs embedded in an electron-dense core (Fig. 4A, D-F). The cross-sectional view of the middle portion of the exocuticle revealed that the pore canals have a nearly circular shape with spokes emanating from the center and consisting of alternating electron-dense and electron-lucent materials at the edges as well as an electron-lucent hub in the middle (Fig. 4F). An electron-lucent spacing at the boundary between the pore canals and the horizontal laminae is also evident (arrows in Fig. 4A, B, D and F).

3.6. Formation of elytral dorsal mesocuticle and endocuticle after eclosion

Dramatic changes occur in the type of cuticular layers being deposited at about the time of adult eclosion. A distinctly different type of cuticle, which is probably the layer referred to as mesocuticle in the beetle literature (Cheng et al., 2009), becomes evident underneath the exocuticle by 1 day after eclosion (1 DAE) in *T. castaneum* (Fig. 5A). The mesocuticle consists of seven to ten horizontal (chitinous) laminae, which are less compact than those in the exocuticle (Fig. 5B). In the mesocuticle the pore canals containing PCFs are less prominent and not as wide but are still connected to the APMPs, which are smaller (Fig. 5A and B). The epidermal cells exhibit evenly spaced microvilli with their tips containing electron-dense plaques (Fig. 5B).

At 2 DAE the apical plasma membrane becomes smooth (Fig. 5C and D). The architectures/shapes of the horizontal laminae and the vertical pore canals containing PCFs in the exocuticle become obscure at 2 DAE possibly due to an increased level of tanning of the rigid adult cuticle including the dorsal elytral cuticle (Fig. 5C and Fig. S2). The outer boundary of the pore canal, in addition, becomes electron-dense, presumably due to deposition of cuticular proteins on the PCF on the outer wall of the pore canal (Fig. 5C and Fig. S2 and see arrows in Fig. 6C). Most prominently, the innermost endocuticle is composed of a thick macrofiber/Balken type of laminae (400–600 nm), which are evident underneath the mesocuticle (Fig. 5C and D).

The endocuticular layer of the elytral dorsal cuticle continues to grow at a rate of ~1 lamina per day (Fig. S2) and by 9 DAE it has reached its maximum size of ~9–10 laminae in the mature cuticle (Fig. 6A and B). The enodcuticular layer together with the mesocuticle accounts for about half of the total thickness of the rigid elytral dorsal cuticle of mature *T. castaneum* (Fig. 6A). Laminae of the endocuticle, in contrast to those of the exocuticle and mesocuticle, appear to be rotated at some angle with respect to one another because several of the vertically oriented narrow pore canals appear to be twisted and traverse discontinuously throughout the layer. Each lamina exhibits a different degree of electron density as well (Fig. 6A and B and Fig. S2). APMPs become smaller and are not obvious at 5 DAE (Fig. 6B and Fig. S2). Immunogold labeling using wheat germ agglutinin (WGA) that binds to chitin indicates that the laminae and pore canals in both mesocuticle and endocuticle contain chitin, which also is found in the exocuticle (Fig. 6D–F).

3.7. Formation of elytral ventral cuticle

Since the elytron contains a membranous ventral cuticle, we also investigated whether the elytral ventral cuticle was developed in synchrony with the rigid dorsal cuticle. Similar to the results observed in the study of elytral dorsal cuticle development, at 54 HAP, the apical plasma membrane in the ventral cuticle is also irregular (arrows in Fig. 7A) and envelope formation is not evident. Electron-dense envelope fragments are formed by 66 HAP (Fig. 7B), and these fuse into a continuous layer at 72 HAP (Fig. 7C). Ventral epicuticle is evident at 84 HAP (Fig. 7D). Interestingly, in contrast to the dorsal cuticle where a relatively thick-forming exocuticle is evident at 90 HAP (Fig. 3C–E), the morphology and thickness of the elytral ventral cuticle has changed very little (Fig. 7E), suggesting that there is no additional ventral cuticle layer/lamina deposition at 96 HAP. At 120 HAP a thin layer (~80 nm), which we denote as “non-laminate exocuticle”, is evident underneath the epicuticle (Fig. 7F). This layer does not show any laminate architecture, and becomes very electron-dense after adult eclosion (Fig. 7G–J). At 1 DAE an endocuticle that is composed of a thick macrofiber/Balken type of laminae (~220 nm) is formed underneath the non-laminate exocuticle (Fig. 7G). As observed in formation of endocuticle of the elytral dorsal cuticle, the endocuticle of the elytral ventral cuticle continues to grow at a rate of ~1 lamina per day and eventually consists of ~9 laminae in the mature cuticle at 9 DAE (Fig. 7H–J). In addition, like the laminae of the innermost endocuticle of elytral dorsal cuticle, those of the elytral ventral cuticle appear to be rotated at some angle because each lamina exhibits a different degree of electron density and morphology (Fig. 7I and J). Unlike the elytral dorsal cuticle, the epicuticular layer lacks a laminar architecture and there are no pore canals containing PCFs in their cores in the exocuticular layer of the elytral ventral cuticle. The thin non-laminate exocuticle layer and thick endocuticle laminae in the elytral ventral cuticle appear to contain chitin (Fig. 7K–M).

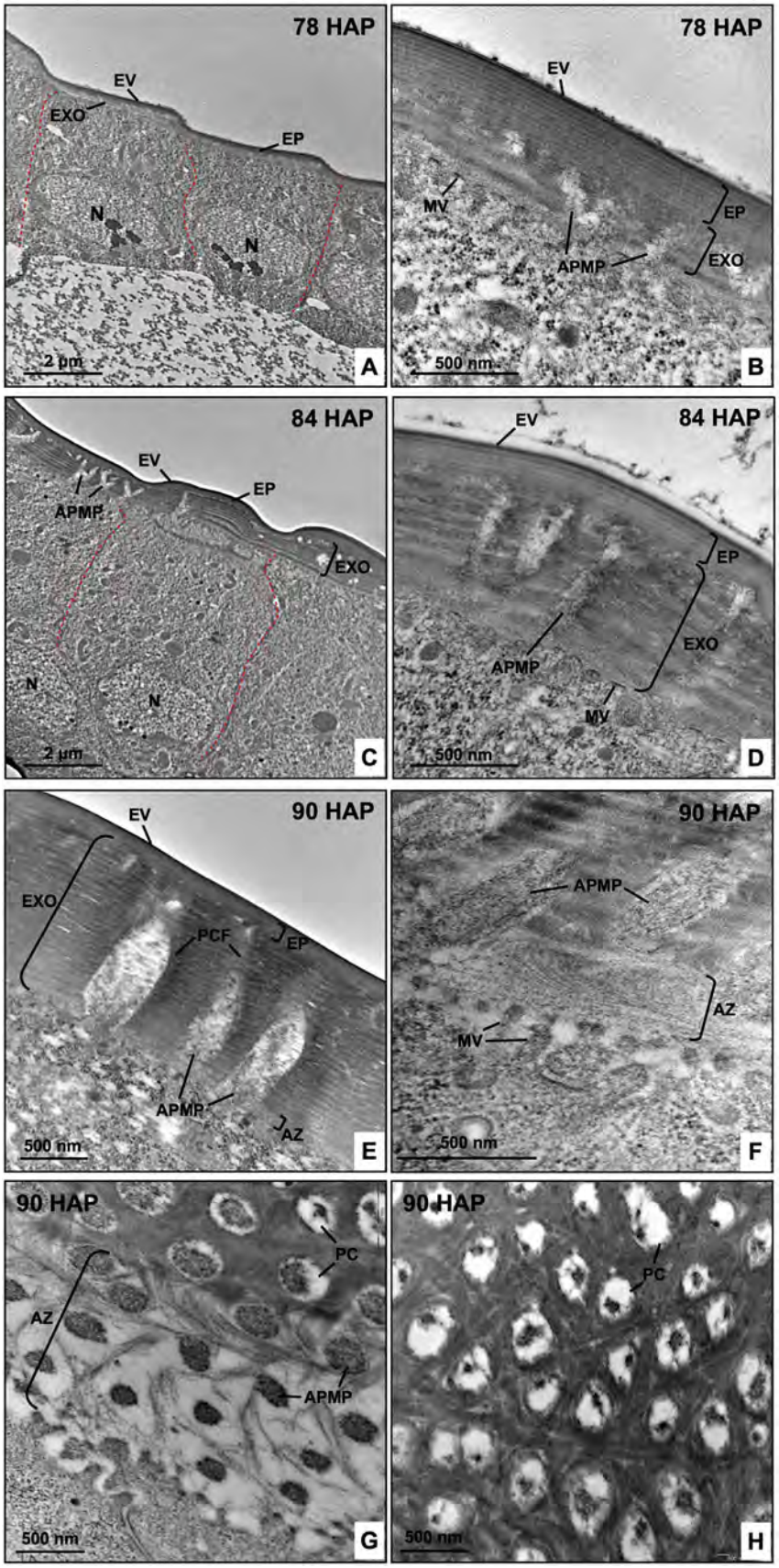

Fig. 3. Ultrastructure of elytral dorsal cuticle at 78 and 90 HAP. (A and B) The envelope and epicuticle appear to be mature and several layers in the latter are evident at 78 HAP. Exocuticle is being formed underneath the epicuticle. The apical plasma membranes protrude throughout the forming exocuticle. Horizontal laminae are present but are not yet organized. (C and D) The exocuticle becomes thicker and horizontal laminae are more compacted at 84 HAP. Pore canals are starting to form around the apical plasma membrane

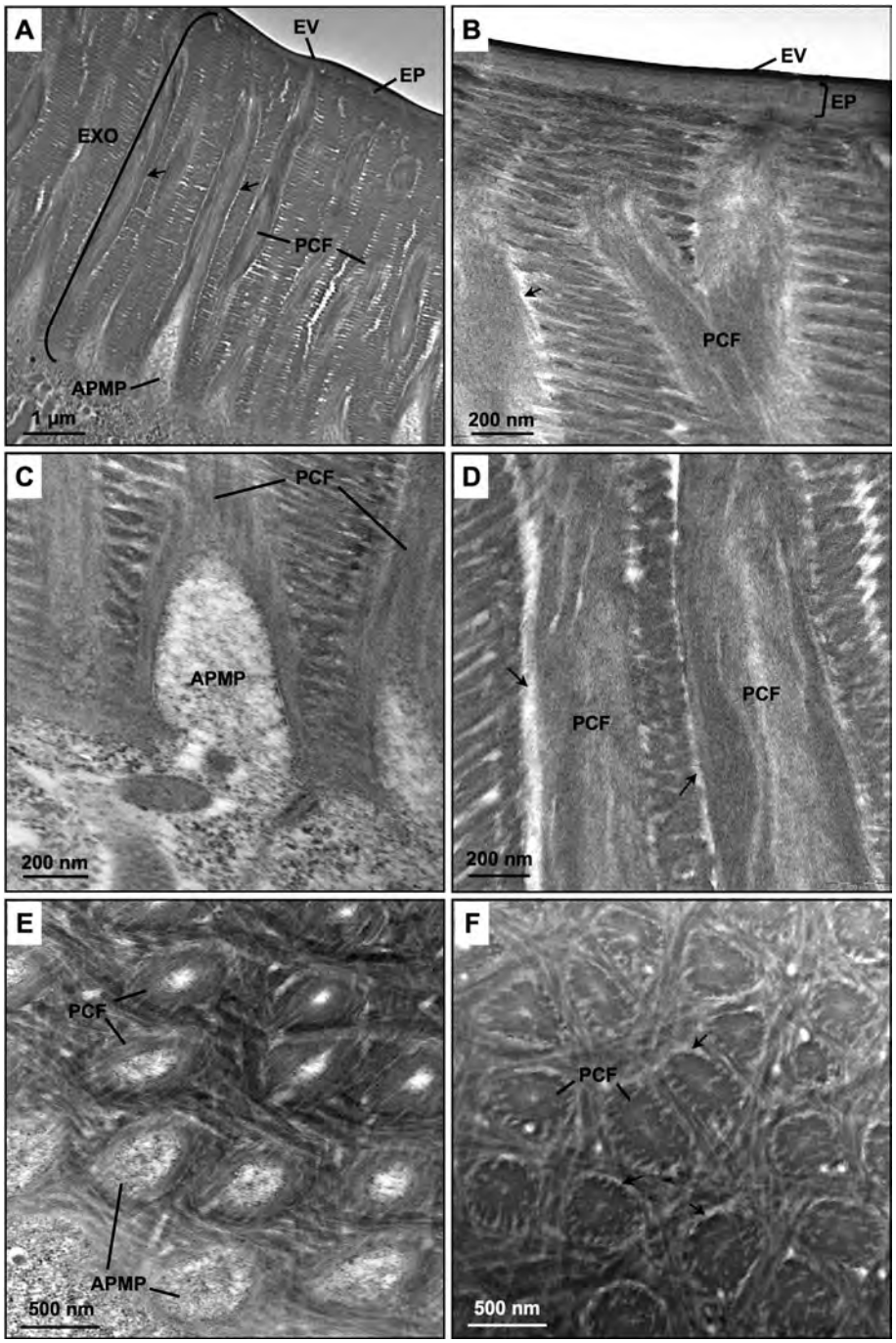

Fig. 4. Ultrastructure of elytral dorsal cuticle of the pharate adult. (A) At the pharate adult stage (120 HAP), large numbers of horizontal laminae are deposited and vertical pore canal fibers are formed in the exocuticle. (B) Pore canal fibers are branched near the epicuticle. Larger magnifications of the inner cuticle region (C) and the pore canal fibers and horizontal laminae (D). Oblique-section of the inner (E) and cross-section of the middle (F) portion of exocuticle. Arrows in A, B, D and F indicate electron-lucent spacing at the boundary between the pore canal fibers and the horizontal laminae. EV, envelope; EP, epicuticle; EXO, exocuticle; PCF, pore canal fiber; APMP, apical plasma membrane protrusion.

3.8. Thickness of layers in elytral dorsal and ventral cuticles in the mature adult

Table 1 lists the thickness values of the various layers in the dorsal and ventral cuticles of the fully developed elytron dissected from 9 d-old *T. castaneum* adults. The dorsal cuticle is about 4-fold thicker than the ventral cuticle. Although the envelope, epicuticle and endocuticle layers are rather similar in thickness in the two cuticles, the exocuticular layer together with the mesocuticular layer, however, are about 80-fold thicker in the dorsal cuticle than

protrusions (APMPs), which traverse the entire exocuticle. (E and F) Chitinous horizontal laminae in the exocuticle are more numerous at 90 HAP. The vertical pore canal fibers appear throughout the forming exocuticle. The assembly zone is present at this time. Oblique-section of the inner (G) and cross-section of the middle (H) portions of the exocuticle reveal electron-lucent pore canals containing electron-dense materials in their cores. EV, envelope; EP, epicuticle; EXO, exocuticle; N, nucleus; MV, microvilli; APMP, apical plasma membrane protrusion; PCF, pore canal fiber; AZ, assembly zone; PC, pore canal.

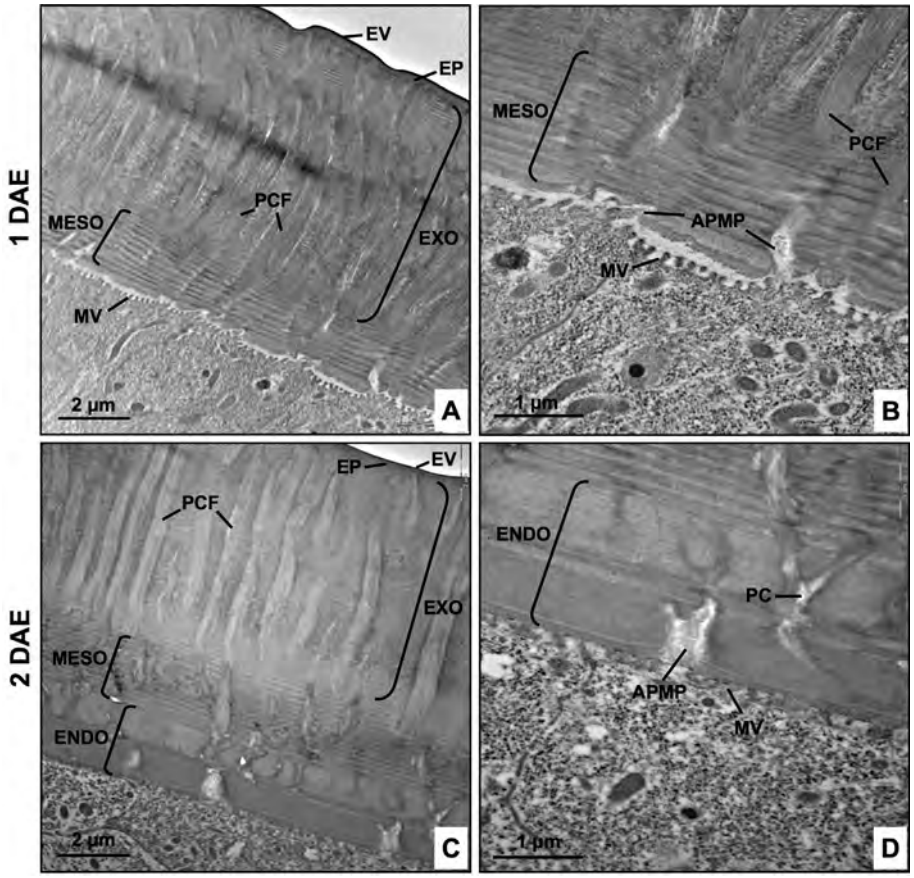

Fig. 5. Mesocuticle and endocuticle formation in elytral dorsal cuticle after adult eclosion. (A and B) An additional layer called the mesocuticle, which consists of less compacted horizontal laminae, is deposited under the exocuticle at 1 d post eclosion (1 DAE). Pore canals consisting of pore canal fibers become smaller and narrower. Evenly spaced microvilli and small apical plasma membrane protrusions are evident. (C and D) Endocuticle composed of thick brick-like horizontal laminae are formed at 2 DAE. Pore canals are evident in the layers. EV, envelope; EP, epicuticle; PCF, pore canal fiber; EXO, exocuticle; MESO, mesocuticle; MV, microvilli; ENDO, endocuticle; PC, pore canal.

the nonlaminated exocuticular layer in the ventral cuticle. The thickest laminae occur in the endocuticular layer of the dorsal cuticle and the thinnest occur in the exocuticular layer. The dorsal cuticle contains pore canal fibers with a diameter of nearly 0.5 μm, whereas the ventral cuticle does not.

4. Discussion

The elytron develops in a carefully orchestrated manner in which the multiple cuticular sublayers appear sequentially. The time-course of development of the cuticle with its complex array of sublayers has been studied previously using primarily soft cuticles of the developing embryo and wing of *D. melanogaster*, the larval cuticle of the larger canna leafroller moth, *Calpodes ethlius*, and the second ventral abdominal sclerite cuticle of the boll weevil, *Anthonomus grandis*, during the fifth instar as the weevil transitions into pupa and during the period that spans adult eclosion (Leopold et al., 1992; Locke, 1966; Moussian et al., 2006; Sobala and Adler, 2016). While the first three examples represent soft cuticle, the third one represents a more rigid one. These studies have revealed that the order of deposition of the multiple sublayers roughly parallels their structural order starting from the outside and proceeding towards the apical surface of the underlying epidermal cells that secrete the cuticle. The envelope layer appears first and is followed by the epicuticle and then by the procuticle. Subtle differences were noted between dipteran (*D. melanogaster*) cuticle development and that of the cuticles in a lepidopteran (*C. ethlius*) and coleopteran (*A. grandis*). Our results are consistent with the hierarchical appearance and regulation of gene expression in cuticular layers reported during wing cuticle development in *D. melanogaster* (Sobala and Adler, 2016).

4.1. Envelope is the first layer to be deposited

Moussian et al. (2006) have reported that the *D. melanogaster* envelope consists of one electron-lucid layer spaced between two electron-dense layers at stage 15, which is the early cuticle development stage of embryogenesis. Subsequently, when the envelope is fully matured at stages 17b to 17c, there are five alternating electron-dense and -lucid sheets. With *T. castaneum*, two electron-dense layers with an electron-lucent layer in between are observed in the early stages of cuticle development, a result similar to the *C. ethlius* larval envelope, which is also trilaminate (Locke, 1966). In *T. castaneum*, a fragmented envelope is produced at the tip of the apical plasma membrane at 60 HAP and the envelope fragments become fused to form a continuous layer at 66 HAP. This observation is also consistent with envelope development in *D. melanogaster* reported previously, in which fragments of envelope are first produced at the tips of microvilli in the apical plasma membranes, which then fuse to form a continuous layer of envelope (Moussian, 2010; Moussian et al., 2006).

4.2. Epicuticle layer is deposited next

In the adult weevil of *A. grandis*, a dense epicuticle was reported below the envelope layer (named outer epicuticle) by Leopold et al.

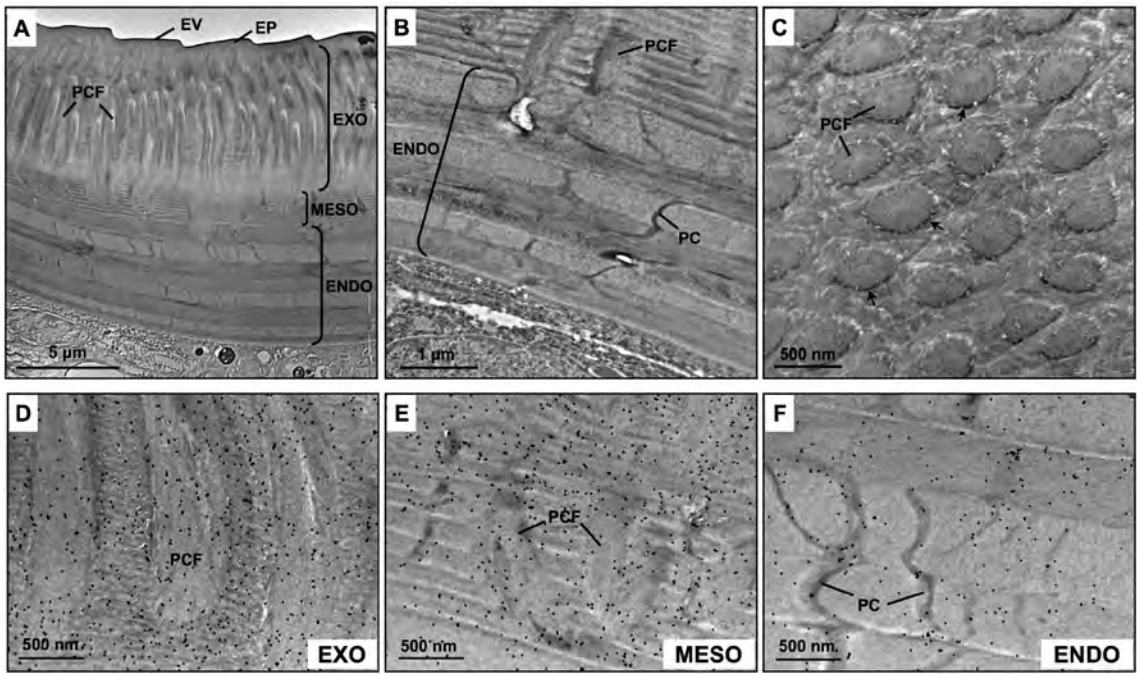

Fig. 6. Ultrastructure of elytral dorsal cuticle in the mature adult. (A) More endocuticular laminae (~9 laminae) have been deposited at 9 d after eclosion (9 DAE). (B) Magnified images of the endocuticle. Pore canals traverse and twist throughout the endocuticle. (C) Cross-section of the middle portion of the exocuticle. Arrows indicate the electron-dense outer boundary of the pore canal. Immunogold-labeling of chitin in exocuticle (D), mesocuticle (E) and endocuticle (F) using wheat germ agglutinin (WGA). EV, envelope; EP, epicuticle; EXO, exocuticle; MESO; mesocuticle; ENDO, endocuticle; PCF, pore canal fiber; PC, pore canal.

(1992). Also in *C. ethlius*, synthesis of the epicuticle begins after completion of the envelope and most likely before deposition of the procuticle (Locke and Huie, 1979). Similarly, in *T. castaneum*, the epicuticle with multiple laminae is formed around 72 HAP after the completion of the envelope layer (at 66 HAP), but before the exocuticle appears at 78 HAP. Chitin is undetectable in the epicuticle of *T. castaneum* by WGA-gold labeling and electron microscopy (Noh et al., 2015) and that result is consistent with the hypothesis that the epicuticle/envelope does not significantly influence the rigidity of the cuticle (Andersen, 1977; Cheng et al., 2009; Neville, 1975). The identity of proteins, if any, in the epicuticle is unknown. Several of the structural proteins of the cuticle with the Rebers & Riddiford consensus sequence have been localized exclusively in the procuticle but not in the envelope or epicuticle (Noh et al., 2014, 2015; Vannini and Willis, 2017). Tanning reactions apparently begin in the epicuticle. It is not yet established whether the tanning phenoloxidase, laccase 2 (Arakane et al., 2005), is located in this layer. Thus, the physiological function of the epicuticular layer in the process of tanning remains to be determined.

4.3. Exocuticle and pore canals develop synchronously

The innermost cuticular layer or procuticle (exocuticle and endocuticle) is generated as stacks of laminae, which contain chitin and several chitin-binding proteins (Dong et al., 2016; Togawa et al., 2004; Willis et al., 2012). In *T. castaneum*, deposition of the procuticle starts around 78 HAP and continues for several days after adult eclosion. Chitin is synthesized and extruded by chitin synthase located on the apical plasma membrane of epidermal cells and self-assemble in the assembly zone to form chitin nanofibrils. These nanofibrils are assembled with the assistance of several proteins into loosely packed laminae of the nascent exocuticle, which becomes more dense and compacted as the fibrils move outward (Fabritius et al., 2009; Muthukrishnan et al., 2016; Nikolov et al., 2010). During the early period of exocuticle deposition, the apical plasma membrane penetrates into the newly forming exocuticle at regular intervals. At this stage, these apical plasma membrane protrusions (APMPs) reach the bottom of the epicuticle, indicating that they also may contribute materials for the growth of the epicuticle. Rudimentary pore canals can be observed as early as 90 HAP when they exhibit a core of electron dense material surrounded by loosely packed chitin fibers. At 120 HAP both the exocuticle and pore canals grow simultaneously. APMPs appear to be confined to the lower portion of the procuticle at all times. The high density of pore canals containing PCFs, together with their size and bifurcation as they approach the epicuticle, suggests a mechanical-support role for these structures in the dorsal cuticle. As they mature, the pore canals assume a more orderly structure as shown in cross sections (Fig. 4F), indicating a central electron-lucent column and a surrounding circular colonnade of thick electron-dense peripheral fibers. It should be noted that the pore canals in *T. castaneum* do not follow a helical path in the exocuticle as has been observed in rigid cuticles of some other beetle and lobster species (Cheng et al., 2008, 2009; Leopold et al., 1992).

4.4. Development of the endocuticle

Even though the procuticle continues to grow after adult eclosion, it has an entirely different shape, compared to the orderly laminar exocuticle or the mesocuticle. In *T. castaneum*, the endocuticle begins to form about 1 d after adult eclosion (1 DAE) and has a brick (macrofiber/Balken)-type structure that is periodically disrupted by the pore canals that traverse it vertically. These thick layers are deposited at a rate of almost one layer per day until around 9 days after eclosion. Brick-like laminae in endocuticle appear to be a common structure of the rigid cuticle in beetles (van de Kamp et al., 2016). These brick-like structures are similar to the "pseudo-orthogonal plywood" structures in cuticles of *A. grandis*

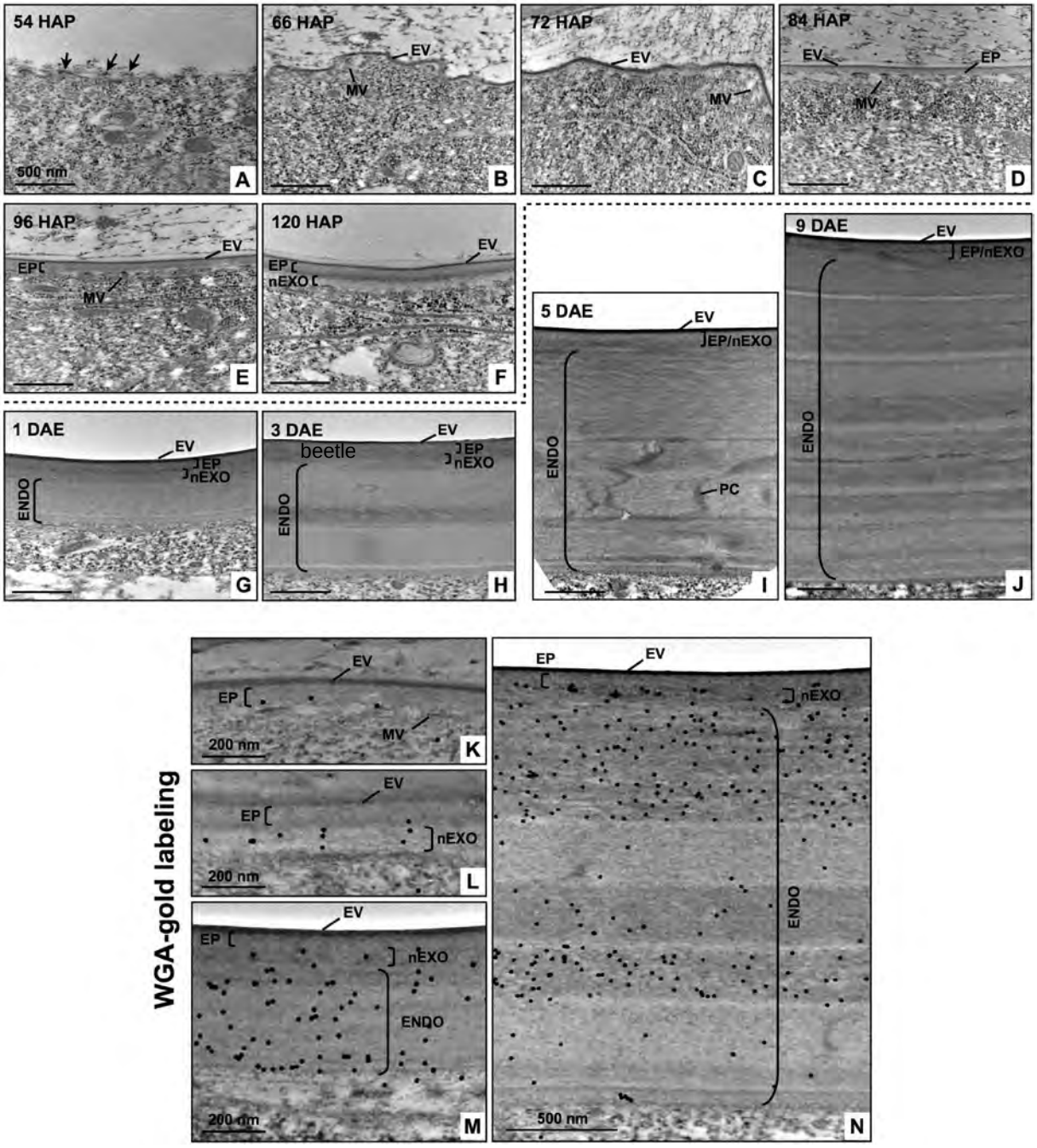

Fig. 7. Ultrastructure of elytral ventral cuticle during adult development. The apical plasma membrane of epidermal cells is irregular (arrows in A), and envelope formation has not yet occurred at 54 HAP (A). The envelope fragments are formed at the tip of apical plasma membrane at 66 HAP (B) and they are fused to form a continuous layer, which completely covers the top of the entire epidermis at 72 HAP (C). The epicuticle has been being formed underneath the envelope by 84 HAP (D), and the morphology and thickness of this region do not change significantly by 96 HAP (E). A thin electron-lucent layer (non-laminated exocuticle, nEXO) is evident at 120 HAP (F), which becomes electron-dense after adult eclosion. Endocuticle composed of thicker brick-like laminae is formed under the non-laminated exocuticle layer at 1 DAE (G) and thickens by 9 DAE (H and J). Immunogold-labeling of chitin in the elytral ventral cuticles at 96 HAP (K), 120 HAP (L), 1 DAE (M) and 5 DAE (N) was performed using wheat germ agglutinin (WGA). EV, envelope; MV, microvilli; EP, epicuticle; ENDO, endocuticle; PC, pore canal.

and the Japanese beetle, *Popillia japonica* (Cheng et al., 2008; Leopold et al., 1992; Zelazny and Neville, 1972). In those structures, successive unidirectionally oriented thick laminae stack upon each other either orthogonally or at smaller angles (30–90°), presumably with small amounts of intervening transitional helicoidal layers between adjacent macrofibers. In the endocuticle of the elytral dorsal cuticle of *T. castaneum*, this angle appears to be approximately 60° because every fourth lamina exhibits a similar degree of electron density as well as morphology.

4.5. Development of pore canals

Pore canal fibers (PCFs) appear to be a unique and important structural feature of arthropod cuticle, which have been implicated in the transport of lipid metabolites to the outermost layers (Delachambre, 1971; Wigglesworth, 1985). Cheng et al. (2009)

Table 1
Thickness of elytral dorsal and ventral cuticular sublayers in *Tribolium castaneum*.

	Cuticle layer	Thickness (μm)
Elytral dorsal cuticle	Envelope	0.033 ± 0.017
	Epicuticle	0.241 ± 0.099
	Exocuticle	6.710 ± 1.101
		(each lamina: 0.058 ± 0.004)
	Mesocuticle	1.241 ± 0.234
		(each lamina: 0.141 ± 0.020)
	Endocuticle	3.907 ± 0.777
		(each lamina: 0.344 ± 0.029)
	Pore canal fiber	0.416 ± 0.073
		(diameter)
	Entire cuticle	**12.166 ± 1.488**
Elytral ventral cuticle	Envelope	0.032 ± 0.007
	Epicuticle	0.107 ± 0.016
	Non-laminated exocuticle	0.098 ± 0.018
	Endocuticle	2.611 ± 0.600
	Entire cuticle	**2.902 ± 0.642**

reported that pore canals in the beetle, *P. japonica*, and in the exoskeletons of two crustaceans, *Callinectes sapidus* (Atlantic blue crab) and *Homarus americanus* (American lobster), followed a twisted ribbon-like path through the exocuticle. However, in the elytron of *T. castaneum*, the pore canals in the exocuticle are far more numerous and wider, and do not appear to follow a helicoidal path. A similar lack of helicoidicity of pore canals in the exocuticle has been reported previously in the mealworm, *Tenebrio molitor* (Delachambre, 1971). Pore canals have been shown by shear-stress measuring experiments to perform structural functions in the lobster exoskeleton and to increase the resistance against delamination (Fabritius et al., 2009). The wide distribution of pore canals containing PCFs in the cross-sectional area of the elytral dorsal cuticle of *T. castaneum* and the specific arrangement of chitin fiber bundles therein is highly suggestive of a major supportive structural role for them.

In *T. castaneum*, PCFs begin to form around 90 HAP directly above the APMPs, suggesting that chitin in these structures arises via chitin synthases (CHSs) embedded in the plasma membrane associated with APMPs that lie perpendicular to the cell surface. CHSs in the APMPs would produce vertically oriented fibers, which would self-associate to form the pore canal fibers. WGA-gold staining has confirmed that these fibers do contain chitin. Initially, the PCFs are not organized into bundles but do become organized into bundles by the pharate adult stage. There are electron-lucent regions between the PCFs and horizontal laminae (arrows in Fig. 4A, B, D and F). Interestingly, these electron-lucent regions are filled with unknown components after adult eclosion, but then they are no longer evident in 2–3 d-old adults. Pore canals containing PCFs are also present in the mesocuticle produced after adult eclosion, but these become progressively thinner with an electron dense core (Fig. 5). The APMPs regress as the cuticle matures and penetrate only into the bottom one or two layers of the mesocuticle or the brick-like endocuticle by 1–2 DAE.

Cuticular proteins in the pore canals may associate with or stabilize the PCFs. Noh et al. (2015) have demonstrated that depletion of a *T. castaneum* cuticular protein, TcCPR4, results in an abnormal morphology of the pore canals including amorphous PCFs in the pore canal lumen, especially when combined with depletion of a second cuticular protein, TcCPR27.

5. Conclusions

Formation and ultrastructure of the thick dorsal and thin ventral cuticles of the elytron are summarized in cartoon form in Fig. 8, and the thickness of each layer in both cuticles is shown in Table 1. Development of the elytral ventral cuticle initially follows that of the dorsal cuticle until the envelope/epicuticle layers are completed (Fig. 8A–C). Subsequently, the morphology and thickness of the ventral cuticle do not change significantly until adult eclosion (Fig. 8D and E). This is in contrast with the dorsal cuticle, which undergoes rapid growth at this stage. By 1 day after eclosion, in addition, sublayers of mesocuticle consisting of 7–10 less compacted horizontal laminae are formed underneath the exocuticle in the elytral dorsal cuticle, while this type of layer is not evident in the elytral ventral cuticle. Only a brick-like endocuticular lamina is being formed at this stage in the ventral cuticle (Fig. 8F). Thus, the pattern of cuticle deposition by the two epidermal layers of the elytron that are physically close to each other is substantially different during the period of exocuticle and mesocuticle deposition. However, endocuticle deposition in both the dorsal and ventral cuticles proceeds in a parallel fashion starting 2 days after eclosion, with respect to both the number and type of laminae being deposited (Fig. 8G). These results indicate that development of the elytral dorsal and ventral cuticles is synchronous only during certain phases of cuticle growth in *T. castaneum*.

The reason for the difference in the development of these two cuticles in the same tissue (the elytron) during the period prior to adult eclosion is unclear at this point. However, because homeotic genes regulate “realizator” genes involved not only in the anterior-posterior but also in dorsal-ventral signaling in insect wing discs (Cohen, 1993; Brook et al., 1996; Tomoyasu et al., 2009; Mohit et al., 2006; Sobala and Adler, 2016), differential regulation/expression of these differentiation-specific genes likely contributes to the temporal and morphological differences found in the development of elytral dorsal and ventral cuticles in *T. castaneum*. The major differences between the elytral rigid dorsal and membranous ventral cuticles of the *T. castaneum* elytron are 1) a four-fold greater thickness of the former; 2) the presence of rib-like veins and bristles in the former; 3) the presence of an exocuticle in the former, which contains numerous horizontal laminae and vertical pore canals with pore canal fibers; 4) the presence of a mesocuticle between an exocuticle and endocuticle in the former; 5) the degree of sclerotization, which appears to be much greater in the former; and 6) the levels of cuticular components such as chitin and protein. For example, there is a major difference in chitin content between rigid vein (high chitin) and membranous (little chitin) portions in the wing of the cicada, *Magicicada cassini* (Gullion and Gullion, 2017). The locust (*Schistocerca gregaria*) wing membrane apparently contains no chitin (Smith et al., 2000). All of these differences no doubt contribute to the greater rigidity of the former and the membranous nature of the latter cuticle. Future research

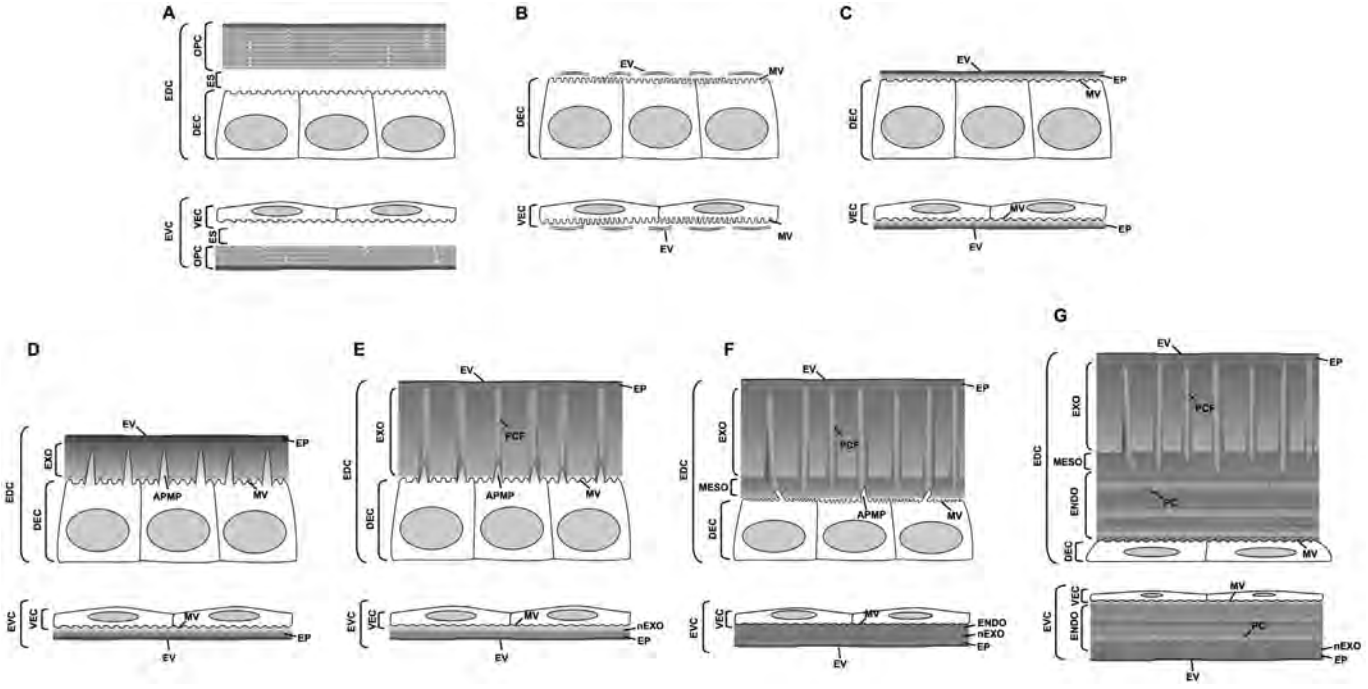

Fig. 8. Schematic diagram of the ultrastructure of elytral dorsal and ventral cuticles during development of *T. castaneum*. (A) Old pupal cuticle (OPC) is separated from the dorsal (DEC) and ventral epidermal cells (VEC) at 24 HAP. (B) The envelope (EV) of the new adult cuticle begins to form discontinuously at the tip of the microvilli (MV) at 60 HAP. (C) Epicuticle (EP) is formed underneath the envelope at 72 HAP. (D) In the elytral dorsal cuticle (EDC), forming exocuticle (EXO) with apical plasma membrane protrusions (APMPs) is observed at 78–84 HAP, while no additional layer/lamina is evident in the elytral ventral cuticle (EVC) at this time. (E) The EXO in the EDC consists of numerous horizontal chitinous laminae and vertically oriented pore canal fibers (PCF) at 120 HAP. A thin electron-lucent layer (non-laminated exocuticle, nEXO) is evident underneath the epicuticle in EVC. (F) Mesocuticle, which is composed of less compacted horizontal chitinous laminae is formed under the exocuticle in EDC by 1 DAE. In contrast, the innermost endocuticular (ENDO) layer consisting of brick-like macrofiber laminae appears to start to form in EVC. (G) The endocuticle continues to grow at a rate of ~1 lamina per day and it consists of ~9–10 laminae in both the mature EDC and EVC at 9 DAE. The laminae of ENDO appear to be rotated at approximately 60°. ES, ecdysial space; PC, pore canal.

should address differences in biochemical composition and the molecular interactions occurring in these two distinct types of cuticle.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ibmb.2017.11.003.

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