

Hierarchical Morphogenesis of Swallowtail Butterfly Wing Scale Nanostructures

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Abstract

The study of color patterns in the animal integument is a fundamental question in biology, with many lepidopteran species being exemplary models in this endeavor due to their relative simplicity and elegance. While significant advances have been made in unravelling the cellular and molecular basis of lepidopteran pigmentary coloration, the morphogenesis of wing scale nanostructures involved in structural color production is not well understood. Contemporary research in this topic largely focuses on a few nymphalid model taxa (*e.g.*, *Bicyclus*, *Heliconius*), despite an overwhelming diversity in the hierarchical nanostructural organization of lepidopteran wing scales. Here, we present a time-resolved, comparative developmental study of hierarchical scale nanostructures in *Parides eurimedes* and five other papilionid species. Our results uphold the putative conserved role of F-actin bundles in acting as spacers between developing ridges, as previously documented in several nymphalid species. Interestingly, while ridges are developing in *P. eurimedes*, plasma membrane manifests irregular mesh-like crossribs characteristic of Papilionidae, which delineate the accretion of cuticle into rows of planar disks in between ridges. Once the ridges have grown, disintegrating F-actin bundles appear to reorganize into a network that supports the invagination of plasma membrane underlying the disks, subsequently forming an extruded honeycomb lattice. Our results uncover a previously undocumented role for F-actin in the morphogenesis of complex wing scale nanostructures, likely specific to Papilionidae.

eLife assessment

This **important** study reports how swallowtail butterflies pattern structures composed of chitin at the nanometer scale to produce structural colors. The work uses state-of-the-art microscopy techniques to **convincingly** show that F-actin is utilized in these butterflies in a novel way to produce structure, paving the way for further studies on growth regulation leading to precise ultrastructures and structural colors.

Introduction

The patterning and coloration of butterfly wings have been a paradigmatic research focus due to their fundamental role in signalling and crypsis (Beldade and Brakefield, 2002; Sweeney et al., 2003; Tsai et al., 2020). Significant advances have been made recently in identifying the cellular and molecular basis of lepidopteran pigmentary coloration (Matsuoka and Monteiro, 2018; Nadeau et al., 2016; Nishikawa et al., 2015; Reed et al., 2011; Van Belleghem et al., 2023; Zhang et al., 2017a). A small number of regulatory genes have been found to exert significant influence on the synthesis and spatial expression of pigments, as well as regulating cuticle deposition, thereby affecting the overall scale morphology (e.g., Banerjee et al., 2022; Brien et al., 2022; Ficarrotta et al., 2022; Matsuoka and Monteiro, 2018; Prakash et al., 2022; Van Belleghem et al., 2023; Wee et al., 2022). For instance, suppression of the *optix* gene has been found to tune lower lamina thickness, inducing iridescent structural coloration and affecting cuticular coverage of the upper lamina (Wasik et al., 2014; Zhang et al., 2017b).

Building on the classic studies on cellular organization of lepidopteran scales (Ghiradella, 1974; Ghiradella, 1985; Ghiradella, 1989; Ghiradella and Radigan, 1976; Overton 1966), a few recent studies utilized advances in microscopy techniques to interrogate the formation of longitudinal ridges (Day et al., 2019; Dinwiddie et al., 2014; McDougal et al., 2021). These insights are, however, limited to ridge formation on the scale surface. Moreover, contemporary research on scale cell development focuses on just a few model taxa (*Bicyclus*, *Precis*, *Heliconius*, *Vanessa*) all from the family of Nymphalidae (Beldade and Brakefield, 2002; Brien et al., 2022; Day et al., 2019; Dinwiddie et al., 2014; Matsuoka and Monteiro, 2018; Nadeau et al., 2016; Pomerantz et al., 2020; Prakash et al., 2021; Reed et al., 2011; Sweeney et al., 2003; Van Belleghem et al., 2023; Wasik et al., 2014; Zhang et al., 2017b), despite an overwhelming diversity in the hierarchical organization of scale nanostructures across Lepidoptera (Ghiradella, 1984; Ghiradella, 1985; Ghiradella, 1989; Prum et al., 2006). A rare exception is the recent work on pierid butterfly *Colias* identifying *bric à brac* as a genetic switch that suppresses UV-reflecting Christmas-tree like ridge gratings in males (Ficarrotta et al., 2022), even though the precise development of the ridge grating is still unresolved (Ghiradella, 1974). Deciphering the cellular and developmental basis of scale organization in beyond just a few model lepidopterans is also highly relevant to current challenges in the synthesis of complex sub-micron and micron-scale hierarchical nanostructures, and could inspire novel biomimetic routes to fabricate multifunctional nanomaterials (Kolle et al., 2010; McDougal et al., 2019; Pokroy et al., 2009; Potyrailo et al., 2007; Siddique et al., 2017; Wilts et al., 2019).

The *bauplan* of lepidopteran wing scales consists an ornamented upper lamina over a relatively unstructured basal lamina, supported by arches with pillar-like struts called trabeculae (Ghiradella, 1984) (Fig. S1). The upper lamina comprised of longitudinal ridges with transverse crossribs framing a set of rectilinear windows. These windows typically open into the interior lumen of the scale cell, but can also be covered by a thin layer of cuticular lamina (Ghiradella, 1984). The sides of the ridges feature microribs – fine flute-like stripes visible at higher magnifications under an electron microscope (Fig. S1).

Among all Lepidoptera families, swallowtail butterflies (Papilionidae) not only encompasses the known diversity of scale nanostructure, but also exhibits some of the most complex hierarchical morphologies found in nature (Ghiradella, 1984; Ghiradella, 1985; Ghiradella, 1989; Prum et al., 2006; Saranathan et al., 2010) (Fig. S1). Despite having a few model species (e.g., *Papilio xuthus*, *P. polytes* and *P. machaon*), the focus of current research on papilionids is limited to molecular basis of mimicry of pigment patterns (e.g., Nishikawa et al., 2015; Yoda et al., 2021). The wing scales of papilionid species (e.g., *Parides sesostris*, *Papilio nireus*) exhibit irregular veins or mesh-like crossribs, often with an underlying honeycomb-like lattice of cuticular walls

enclosing columnar pores (Ghiradella, 1984 [↗](#); Ghiradella, 1985 [↗](#); Ghiradella and Radigan, 1976 [↗](#); Prum et al., 2006 [↗](#); Saranathan et al., 2010 [↗](#); Wilts et al., 2014) (Figs. 1 [↗](#), and S1). In the following, we use “honeycomb lattices” to refer to irregular crossribs with lattice walls extending into the interior lumen (e.g., green scales in *P. sesostris*, *P. arcas* and *P. eurimedes*), and “irregular crossribs” for crossribs that lack such extruded cuticular walls. In any case, these irregular crossribs are an autapomorphy of Papilionid butterflies (Ghiradella, 1984 [↗](#); Ghiradella, 1985 [↗](#); Ghiradella and Radigan, 1976 [↗](#)) that are thought to increase the surface area over which pigments such as papiliochromes can be deposited (Wilts et al., 2012 [↗](#)), and likely play a role in thermoregulation as they are efficient absorbers of solar radiation (Siddique et al., 2017 [↗](#)).

Here, we use scanning electron microscopy (SEM), conventional confocal and super-resolution Structured Illumination Microscopy (3D-SIM) to study the time-resolved development of hierarchical scale nanostructures in wing scales of six papilionid species, focusing mainly on *Parides eurimedes*. We discovered that following ridge formation, rows of chitinous disks form in between the ridges, followed by disintegration of F-actin that reorganizes into a mesh-like network. This network likely acts as a template for subsequent formation of honeycomb lattices by driving plasma membrane invagination into the scale lumen. Our study uncovers a novel process of wing scale nanostructure formation in Papilionid species.

Results

Wing scale ridge formation is conserved in Papilionidae

We comparatively studied the wing scale growth in *Papilio polytes* and *Parides eurimedes*, which have irregular crossribs and honeycomb lattices respectively. Early stages of scale cell growth are as previously documented in several nymphalid species (Day et al., 2019 [↗](#); Dinwiddie et al., 2014 [↗](#); Ghiradella, 1974 [↗](#); Pomerantz et al., 2020 [↗](#)). Wheat germ agglutinin (WGA) stains the plasma membrane during early stages of scale development in *P. eurimedes*. Scale cells from relatively young pupae at 38% development resemble elongated buds containing densely packed polymerizing F-actin filaments (Fig. S2A-A” and B-B”). At 43% development, F-actin filaments form thicker bundles that extend down the full length of scale cells (Fig. S2C”), laying down a scaffold that determines the eventual position of ridges (Dinwiddie et al., 2014 [↗](#)). WGA stains pleating membranes (longitudinal striations) in between adjacent rows of F-actin bundles (Fig. S2C-C” and D-D”). Around 48% to 50% development, the developing ridges can be more clearly discerned in between F-actin bundles (Fig. S2E-E”, S15D-D” and E-E”). At this stage, there also appears to be irregular gaps in WGA staining in between the ridges of *P. eurimedes* scales (Fig. S2E). These vein-like features resemble the irregular crossribs seen in adult wing scales (Fig. 1B-C [↗](#), Fig. S15B-C).

Cross-rib and cuticular disk formation in *Parides eurimedes*

We use lectin and membrane stains to follow plasma membrane topology and cuticle deposition during scale cell maturation (Figs. 2 [↗](#), and S3-S6). At 48% development, plasma membrane appears mottled while WGA predominantly stains longitudinal striations that will become ridges (Figs. 2A-C [↗](#), and S3B-B”). In cross-sections, both stains appear to be in close contact at the periphery of the cell, with plasma membrane underlying the cuticular layer (Figs. 2C [↗](#) and S3C-C”). This observation is expected, as the cuticle of extracellular origin is being deposited onto the scale cell membrane. At 52% pupal development, plasma membrane shows distinct pleating corresponding to the developing ridges, with irregular crossribs in between (Figs. 2A’-C’ [↗](#), and S4A’-C’). The bulk of cuticle remains above the plasma membrane at this stage (Figs. 2C’ [↗](#), and S4C”). At around 62% pupal development, ridges appear to have grown to their near final configuration while lectin staining reveals planar, disk-like cuticular features (hereafter cuticular disks) arranged in rows in between the ridges (Figs. 2A”-C” [↗](#), 2A”-C”, S5 and S6).

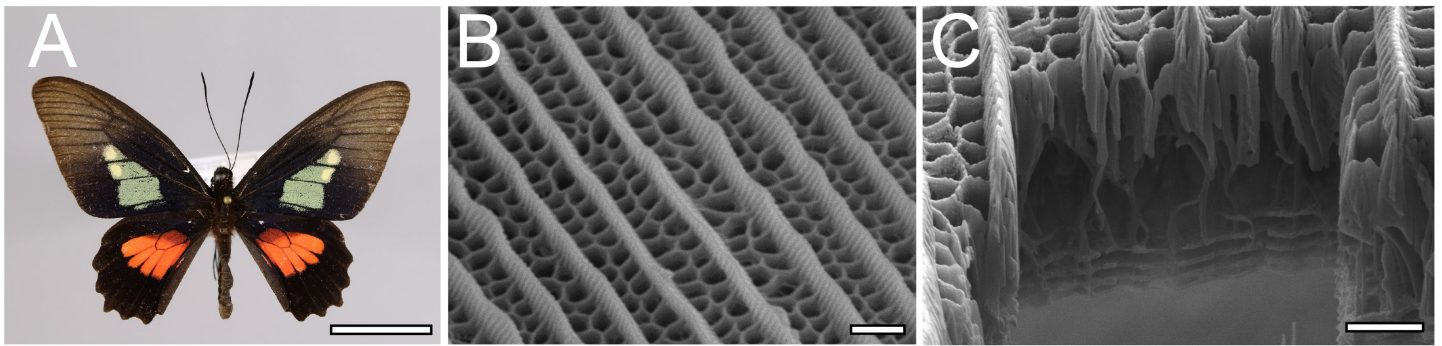


Figure 1.

Hierarchical nanostructure of wing scales in *Parides eurimedes* (Papilionidae). (A) Adult male with structurally-colored green patches on their dorsal forewings. Credit: iDigBio YPM Ent 433579. (B) SEM top-view and (C) FIB-SEM cross-sectional image of an adult green scale showing ridges and honeycomb lattice ending in trabeculae on top of a perforated multilayer lattice. Scale bars: (A) – 1cm, (B and C) – 1 μ m.

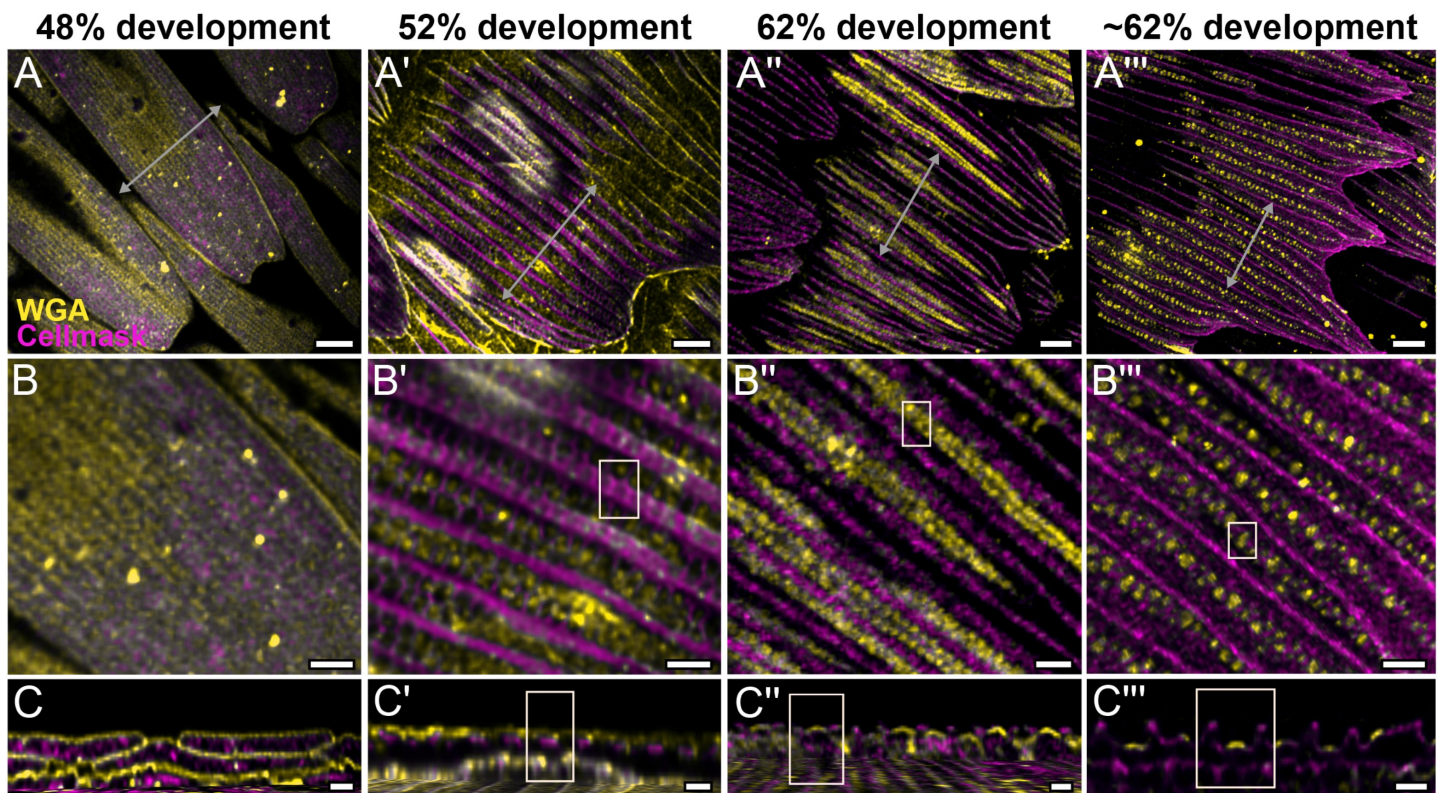


Figure 2.

Morphogenetic time series of the development of cuticular disks in pupal *P. eurimedes* dorsal forewing scales, acquired with a 100x confocal microscope. AF-555 WGA (green) stains chitin and Cellmask (red) stains plasma membrane. By 52% development, Hollow vein-like crossribs appear on plasma membrane in between ridges that serve as a scaffold for cuticle accretion. As scales mature, more cuticle is deposited into these rows of disks bounded by plasma membrane. See also Figs. S4- S6. (B-B''') Close up views of A-A'''. (C-C''') xz cross-sections of the scale at locations marked with grey lines in A-A''' reveal the planar aspect of the cuticular disks. Yellow ROI in C'-C''' correspond to those in B'-B'''. Scale bars (A-A''') – 5µm, (B-B''' and C- C''') – 2µm.

Dynamics of F-actin reorganization in *Parides eurimedes*

We also co-stain chitin and F-actin on the developing pupal scales to investigate the role of cytoskeleton in scale nanostructure formation. Interestingly, as cuticular disks of various sizes are forming in between ridges at 57% development, F-actin bundles are breaking down into short fibrils distributed around the disks (**Figs. 3B-C**, and S7 and movie S1). As the scales became more sclerotized and flattened out later in development (at 67%), disintegrating F-actin appears to reorganize and associate more clearly with cuticular disks, which now appear elongated and slightly tubular in cross-sectional view (**Figs. 3A'-C'**, S8 and movie S2). By 76% development, the cuticular disks appeared hollow, and are surrounded by distinct bulb-like structures formed by the disintegrated F-actin bundles (**Figs. 3A''-C''**, S9 and movie S3).

To better understand the mechanism behind F-actin reorganization, we investigated whether the actin-related proteins, Arp2/3 complex, is a potential factor regulating the dendritic growth of actin fibrils (Hudson and Cooley, 2002; Pollard, 2007; Rouiller et al., 2008). At around 52% development in *P. eurimedes*, Arp2/3 complex appear in a sparse punctate pattern while the F-actin bundles are still intact (**Figs. 4A-C**, and S10). As F-actin bundles disintegrate and start to reorganize around 62% development, a relatively higher density of punctate Arp2/3 complex is seen in close association with the F-actin network (**Figs. 4A'-C'**, and S11). At a later stage (~76% development), no distinctive punctate patterns can be discerned (**Figs. 4A''-C''**, and S12), which is expected given that the reorganization of F-actin is nearly complete by this stage (see **Fig. 3**). However, the scales are more mature at this point with prominent ridges, and autofluorescence signal from the cuticle has shifted into the green wavelengths (Fig. S13), complicating the interpretation of the Arp2/3 immunofluorescence signal.

Pharmacological disruption of Arp2/3 using CK-666

In order to mechanistically test the putative role of Arp2/3 in reorganizing F-actin during honeycomb lattice formation, we carried out pharmacological disruption experiments in pupae of both *Parides* and *Papilio* species using CK-666, a known inhibitor of the Arp2/3 complex (Henson et al., 2015). Unfortunately, we were only able to obtain a small number of *Parides* pupae ($N = 15$), which were already somewhat mature when they arrived, and further, a third of the pupae were dead on arrival (Table S1). Honeycomb lattices can be seen in scales from emerged males of both *P. eurimedes* and *P. iphidamas* pupae treated with CK-666 (Fig. S14). However, the height of the honeycombs in scales from pupae treated on 52% development were on average lower than the control (**Figs. S14A and D**). Furthermore, in *P. eurimedes*, the height of the honeycombs in scales from pupae injected at 57% development were higher on average than those treated at 52% development, but indistinguishable from any controls (**Fig. S14A**). These results are reminiscent of a previous study utilizing cytochalasin-D to inhibit linear actin polymerization (Dinwiddie et al., 2014) that found that once the pupae were past a certain developmental stage, ridge formation is no longer disrupted by the inhibitor. However, despite this intriguing trend in our results being consistent with a hypothesized role for Arp2/3, we require data with robust sample sizes and younger time points (i.e. 28%-48% development) for *Parides* spp. in future experiments to validate the role of Arp2/3.

We also injected CK-666 into the wing pouches of the more readily available *Papilio polytes* pupae from 40%-80% development to cover the possible timeframe during which Arp2/3 complex could be involved in F-actin reorganization. However, neither cuticular disks nor the dendritic reorganization of linear F-actin bundles are observed in *P. polytes*, whose adult scales have irregular crossribs without the deep lattice walls, i.e., no honeycombs (**Fig. S15B and C**). Shortly after ridge formation at 60% development (**Fig. S15 D-D''**), irregular crossribs are seen in between intact F-actin bundles (**Fig. S15 F-F''**). At 70% development, strong chitin autofluorescence signal is seen at the ridges (**Fig. S15 H-I**). Since F-actin reorganization is absent in this species, Arp2/3 inhibition did not disrupt ridge and crossrib formation in *P. polytes* as expected (**Fig. S16**).

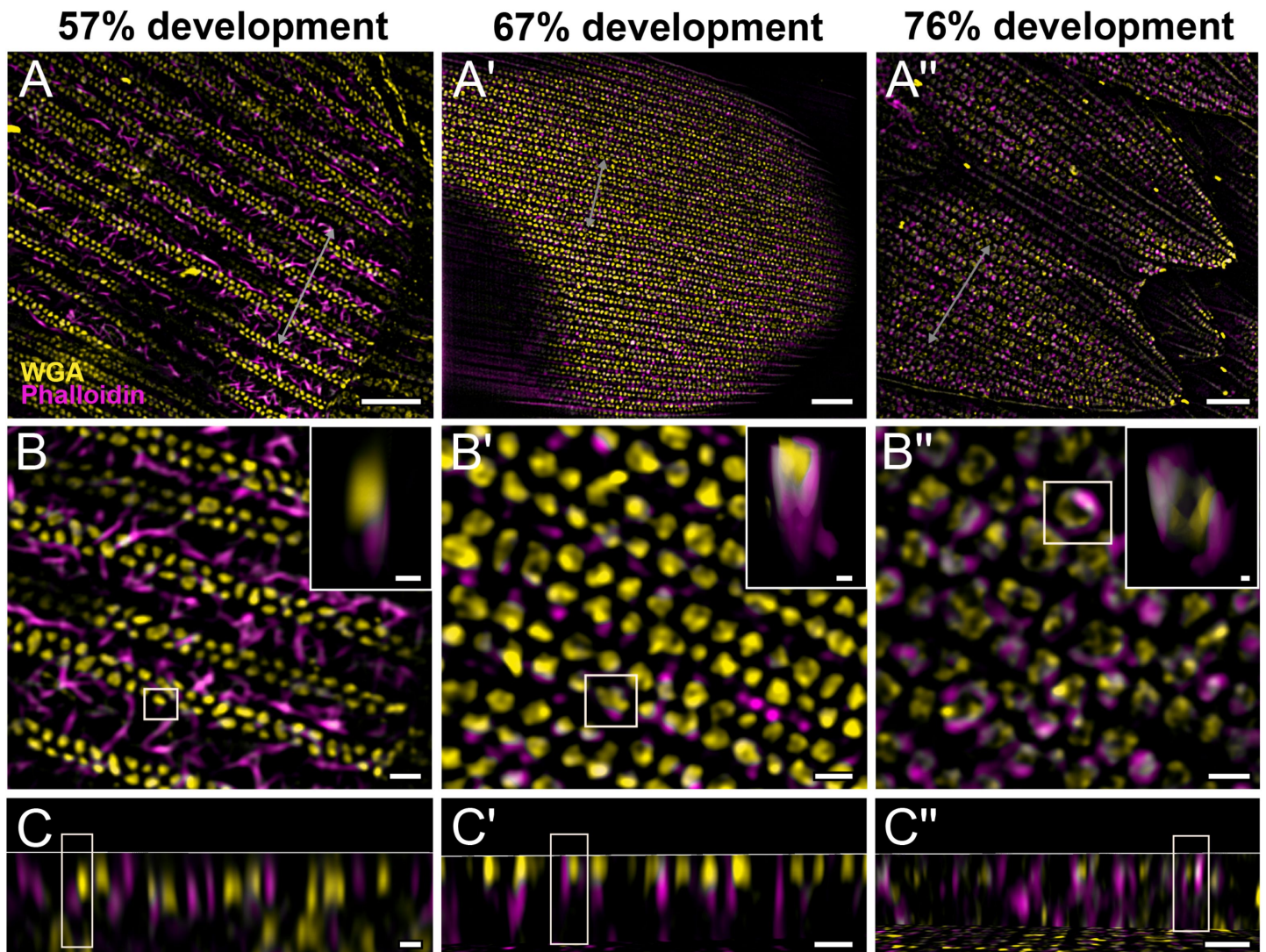


Figure 3.

Morphogenetic time series of the development of columnar honeycomb lattice in pupal *P. eurimedes* dorsal forewing scale cells acquired with super-resolution lattice SIM. AF-555 WGA (green) stains chitin and AF-647 phalloidin (red) stains F-actin. AF-555 WGA show a gradual evolution of the cuticular disks from filled-in planar to hollow tubular outgrowths. The disintegrating F-actin bundles show evidence of reorganization from linear to reticulated features with ring-like cross-sections. See also Figs. S7-S9. (B-B'') Close up views of A-A''. Insets correspond to the regions of interest (ROI) marked in yellow shown with a 3D aspect. (C-C'') xz cross-sections of the scale at locations marked with grey lines in A-A''. Scale bars (A-A'') – 5 μm, (B-B'', C-C'' and insets) – 1 μm.

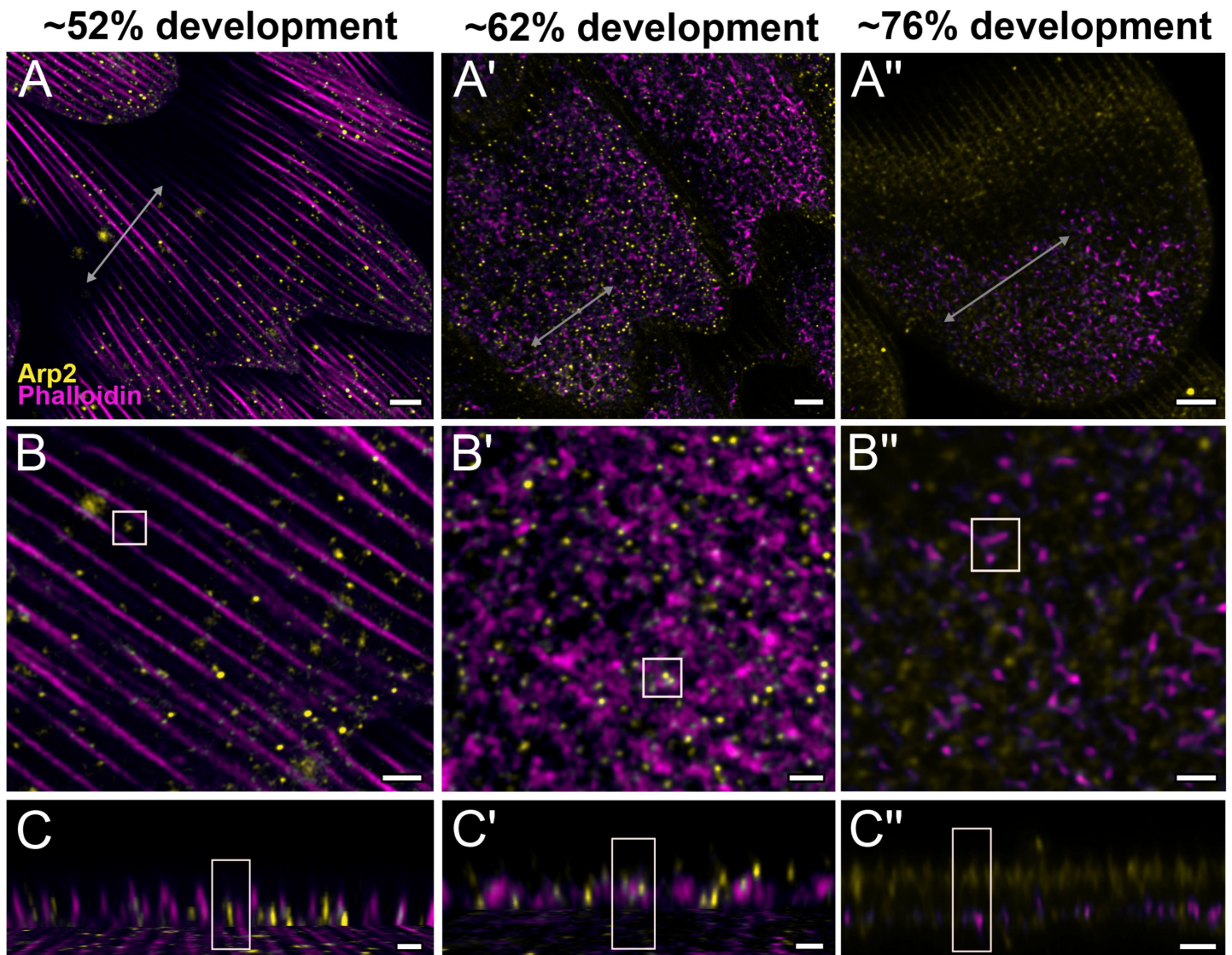


Figure 4.

Arp2/3 complex is involved in F-actin reorganization in pupal *P. eurimedes* wing scales. Dorsal forewing scales stained with AF-594 anti-Arp2 (green) and AF-647 phalloidin (red) acquired with 100x confocal microscope. Initially (~52% development), Arp2/3 complex appear as sparse punctate dots while the F-actin bundles are still intact. As F-actin bundles disintegrate and reorganize, a relatively higher density of punctate Arp2/3 signal is seen in close association with the reticulate F-actin network. Our oldest timepoint in *P. eurimedes* (~76% development) has a large amount of cuticle autofluorescence overlapping with the AF594 signal, but the punctate pattern can be still discerned. See also Figs. S10-S12. (B-B'') Close up views of A-A''. (C-C'') xz cross-sections of the scale at locations marked with grey lines in A-A''. Yellow ROIs correspond to those in B-B''. Scale bars (A-A'') – 5µm, (B-B'' and C-C'') – 2µm.

Concurrent SEM imaging of pupal scales from the same samples show the formation of irregular crossribs is similarly not affected (Fig. S17). Our results suggests the formation of cuticular disks and their subsequent extrusion by reorganizing F-actin is restricted to species with elaborate honeycomb lattice walls such as *Parides eurimedes*.

Honeycomb morphogenesis is conserved across Papilionidae

We also assayed the development of pupal wing scales of several other papilionid species: *Parides arcas*, *Papilio nireus*, and *Papilio palinurus*, (Fig. 5 [↗](#)). Although we were unable to obtain pupae with clearly marked pupation dates for these three species, we observed similar early stages of honeycomb development as seen in *P. eurimedes*. In adult male *P. arcas*, green scales have well developed honeycomb lattice walls that sit above single gyroid crystals (Fig. 5A [↗](#)). During *P. arcas* pupal scale development, both solid and hollow (annular) cuticular disks can be observed in between intact linear actin bundles (Fig. 5B-B' [↗](#) and C-C' [↗](#)). The same is observed in *P. nireus*, where the upper lamina of adult blue scales consists of an elaborate honeycomb lattice with smaller sized pores (Fig. 5D, E [↗](#) and F [↗](#)). In *P. palinurus* adults, green forewing cover scales feature large concave depressions in between the ridges (Fig. 5G [↗](#)). Phalloidin staining of pupal *P. palinurus* scales reveals reorganization of longitudinal F-actin bundles into a shallow concave network (concentric rings in cross-section) (Figs. 5H-I [↗](#), and Movie S4), which closely correspond to the cuticular dimples seen in adult green cover scales.

Discussion

Swallowtail butterflies (Papilionidae) is the sister lineage to all other butterflies (Espeland et al., 2018 [↗](#)) and the showcase family of lepidopterans, as they exhibit some of the most diverse assortment of wing scale nanostructures (Ghiradella, 1985 [↗](#); Prum et al., 2006 [↗](#)). Papilionid wing scales generally exhibit irregular crossribs, with some having underlying cuticular walls enclosing tubular pores, although the regularity and depth of the walls vary between species (Fig. S1). These crossrib patterns are unlike the regular, rectilinear crossribs commonly seen in other lepidopteran families, e.g., Nymphalidae (Fig. S1R). In this study, we extended previous observations that parallel F-actin bundles configure the spacing and position of longitudinal ridges of wing scales (Day et al., 2019 [↗](#); Dinwiddie et al., 2014 [↗](#); Ghiradella, 1974 [↗](#); Pomerantz et al., 2020 [↗](#)) to Papilionidae, further supporting the hypothesis that this mechanism is broadly conserved across lepidopterans.

Our first novel finding is the appearance of irregular crossrib patterns in the plasma membrane between surface ridges, which seemed to serve as a template for chitin accretion into chitinous disks. This process is important as it marks the beginning of honeycomb lattice formation. Further, our results revealed that F-actin plays a predominant and previously unconsidered role in the morphogenesis of some papilionid butterfly wing scales. Once the longitudinal ridges have developed, F-actin bundles, which typically degenerate in nymphalid species (Day et al., 2019 [↗](#); Dinwiddie et al., 2014 [↗](#)), subsequently reorganize into a reticulate network surrounding rows of cuticular disks on the scale surface. This actin network likely draws in plasma membrane underlying the cuticular disks into a porous honeycomb lattice. Similar non-linear F-actin morphologies have been observed in other organisms. In diatoms, F-actin organizes into an interdigitating mesh-like porous network during development (Tesson and Hildebrand, 2010 [↗](#)). This actin network defines frustule (cell walls) morphogenesis by providing a template for silica biomineralization at the meso and micro scales. In mammalian cells, transient ring-like F-actin structures are thought to drive autophagosome generation by serving as a scaffold for mitophagy initiation structures. 3D-SIM revealed F-actin partially associating with mitochondria in the form of curved sheet (Hsieh and Yang, 2019 [↗](#)), akin to the F-actin structures seen in this study.

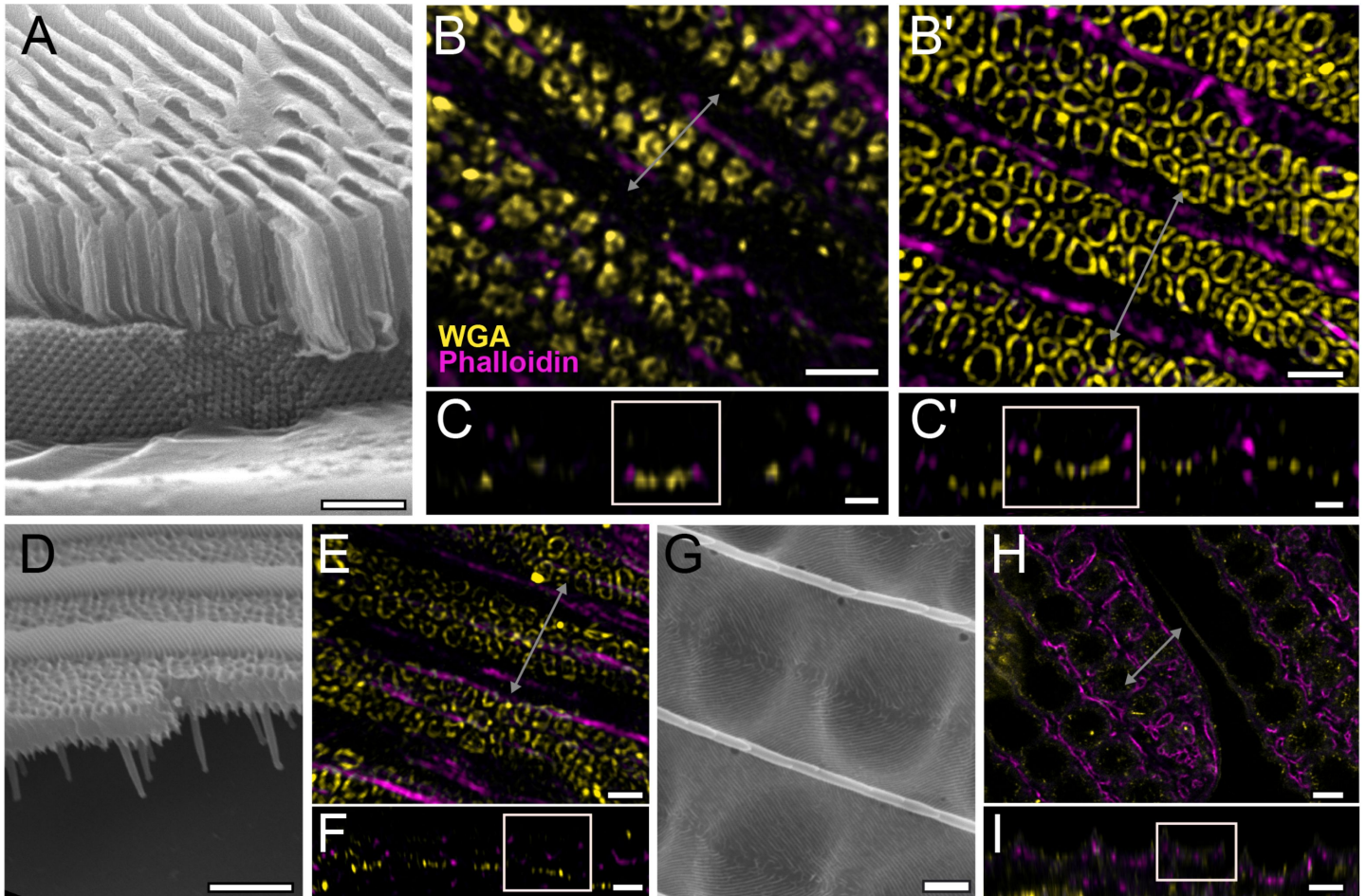


Figure 5.

Conservation of honeycomb lattice development in genus *Parides*. SEM cross-section view of (A) adult green scales in *P. arcas*, (D) blue scales in *P. nireus*, and (G) green scales in *P. palinurus*. All pupal dorsal forewing scales are stained either with AF-555 WGA or FITC-WGA (green) showing chitin, and AF-647 phalloidin or TRITC-phalloidin (red) showing F-actin. (B-C) Maximum projected 3D-SIM micrograph of green cover scales in pupal male *P. arcas* featuring planar cuticular disks similar in shape and arrangement to *P. eurimedes*. (B'-C') Maximum projected 3D-SIM micrograph of green cover scales of a different male *P. arcas* pupa showing irregular crossribs patterns. (E-F) Maximum projected 3D-SIM micrograph of blue cover scales of pupal *P. nireus*, similarly with crossribs and intact linear actin bundles. (H-I) 60x confocal micrographs of green cover scales of pupal *P. palinurus* shown at two different z-planes. A concave network of F-actin underlie the cuticular dimples. At lower z, the actin rings (in cross-section) are smaller in size and show a foam-like appearance. (C-C', F, and I) xz cross-sections of the scales with ROI at locations marked with grey lines in B-B', E, and H respectively. Scale bars (A, D, and G) – 5µm, (B-B', C-C', E, and F) – 1µm, (H-I) – 5µm.

The schematic in **Figure 6** illustrates our proposed formation of honeycomb lattices in some Papilionid wing scales. During early stages of scale formation, F-actin bundles prefigure the loci of future ridge formation in between adjacent actin bundles (Dinwiddie et al., 2014). Plasma membrane then forms a scaffold resembling the irregular crossrib pattern. The cuticle accretes into roughly circular disks delineated by the scaffold. Reorganization of F-actin around the chitinous disks draws the plasma membrane along with the overlying cuticle inwards, moulding it into the lengthened cuticular walls of the honeycomb lattice.

In our attempt to unravel the molecular players in actin reorganization, we used CK-666 to inhibit the role of Arp2/3 complex in actin branching (Henson et al., 2015). However, CK-666 did not quite disrupt the formation of honeycombs even when administered across multiple timepoints. A variety of reasons could potentially explain this. While 100uM of CK-666 has been shown to work in other animal models (Henson et al., 2015), it might still not be a sufficient titre for papilionid butterfly pupal scales. Unfortunately, due to logistical difficulties in obtaining and rearing *Parides spp.*, we were unable to perform a thorough optimization using CK-666. Furthermore, the *Parides* pupae were likely too mature at the time of CK-666 treatment, when the F-actin network has already been established (52% development). In a recent study (Sakamoto et al., 2018), CK-666 did not disrupt the formation of cortical actin meshwork in Sertoli cells of mouse seminiferous tubules, as they were found to be regulated by formins rather than Arp2/3. This suggests that other molecular players, including formins, could be involved in wing scale honeycomb morphogenesis and needs testing.

We note that the irregular crossrib patterns are in place while the ridges are still growing (**Fig. 2A'-C'**). These crossribs appear to constrain and delineate the accumulation of cuticle into planar disks in between the ridges. A close examination of SEM images (**Figs. 1B** and **S1**) reveals that the endpoints of crossribs at the ridge interface are often connected to microribs present on the ridges. Given their relatively small size and pitch, we are unable to resolve the development of microribs here, which likely requires imaging techniques with even higher resolution such as Photo-activated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM). However, both the periodic stripes (microribs) and quasi-periodic spots (crossribs) are reminiscent of Turing patterns (Meinhardt, 1982; Turing, 1952), i.e., implying that an activator-inhibitor type mechanism could be involved in their formation. Alternatively, the crossrib pattern could be produced as a result of spontaneous processes like Ostwald ripening, phase separation, and/or biomechanical forces determined by the mechanical properties of the membranes, leading to stable, quasi-periodic, foam-like perforations on the plasma membrane in between ridges (Dinwiddie et al., 2014; Elson et al., 2010; McDougal et al., 2021; Rosetti et al., 2017; Voorhees, 1985; Wilts et al., 2019).

Overall, the presence of chitinous disks followed by F-actin reorganization appears to be specific to species with extended honeycomb lattice walls, e.g. *Parides eurimedes*, *Parides arcas*, and *Papilio nireus* (Fig. S1A and S1E). Such reorganized actin structures are not observed in species with shallow irregular crossribs, such as *P. polytes* (Fig. S15). On the other hand, several *Papilio* species (e.g., *palinurus*, *blumei*, *karna*) possess multi-layered cover scales (Ghiradella; Prum et al., 2006; Trzeciak et al., 2012) with widely spaced and reduced (in both height and number) ridges. These scales have no apparent crossribs, trabeculae or honeycombs. Instead, they have a characteristic inter-ridge array of concave depressions and underlying perforated lamellae in the scale interior (Fig. S1L and M). Based on our observations, we speculate that the dimpled appearance of cover scales is templated by reorganization of F-actin bundles into a whorl-like concave network. This could represent an extreme modification of the proposed developmental program of the usual papilionid honeycomb lattice. Without crossribs, the pore sizes of honeycombs are likely constrained only by the pitch of the ridges. This is consistent with our observation that the outermost F-actin rings possess dimensions approaching the inter-ridge distance (**Fig. 5C**). Without the trabeculae, the lumen multilayer fills the entire interior of the scale right up to the shallow dimples.

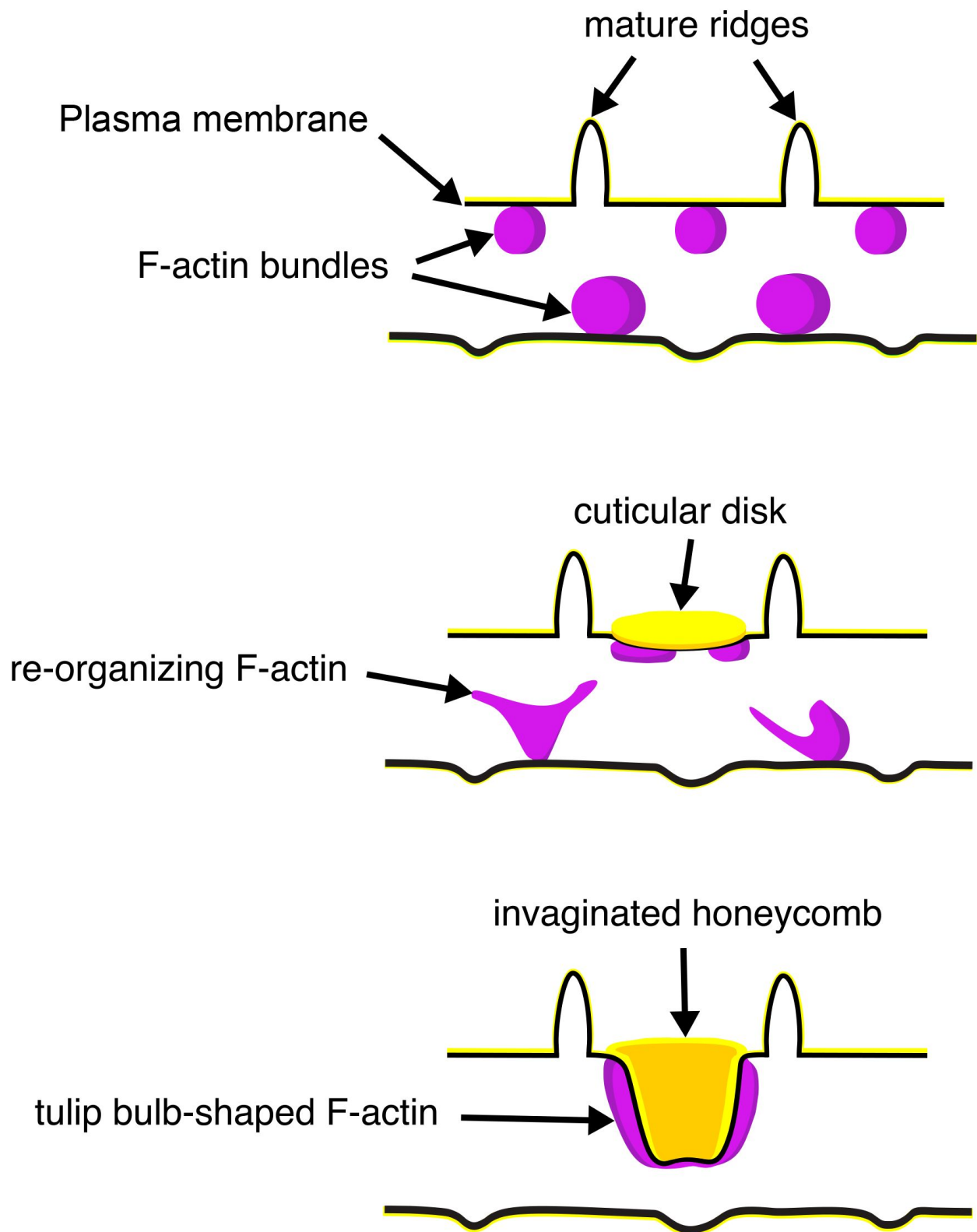


Figure 6.

A schematic illustrating the proposed morphogenesis of honeycomb lattices in papilionid wing scales. During early stages, F-actin bundles prefigure the loci where ridges form in between adjacent actin bundles. Next the plasma membrane (black lines) forms a scaffold where cuticle (green lines and structures) accumulates into the spaces and forms irregular disk-like structures. Reorganization of F-actin around the disks subsequently extrudes the plasma membrane along with the deposited cuticle, forming the honeycomb lattice walls.

Interestingly, in typical lepidopteran scale cells, the trabeculae extend downwards from the crossribs and form columns of arches in between the ridges (Ghiradella, 1974 [↗](#); Ghiradella, 1985 [↗](#); Ghiradella, 1989 [↗](#); Ghiradella and Radigan, 1976 [↗](#); Overton 1966 [↗](#)), suggesting they are developmentally connected. Recently, Matsuoka and Monteiro (2018) [↗](#) found that *DDC* mutant of *Bicyclus anynana* (Nymphalidae) possessed irregularly-spaced and thin crossribs with sheet-like vertical trabeculae instead of feet-like, arched trabeculae (see Fig. 4A [↗](#) of (Matsuoka and Monteiro, 2018 [↗](#))). These mutant scale morphologies are somewhat reminiscent of the irregular crossribs and honeycomb lattice walls of Papilionidae. This suggests spatio-temporal changes in expression patterns of single genes such as *DDC* could possibly alter crossrib morphology and drive honeycomb formation. However, any putative pleiotropic role of pigment-pathway genes in organizing papilionid scale morphology should be reconciled with that of actin reorganization. Future studies could look at knocking out *DDC* and other pigment-pathway genes during papilionid pupal development, in addition to inhibiting actin-binding proteins including Arp2/3 and formins, and tinkering with master regulatory genes like *optix* (Banerjee et al., 2022 [↗](#); Wasik et al., 2014 [↗](#); Zhang et al., 2017b [↗](#)).

The smooth endoplasmic reticulum (SER) has been implicated in templating luminal scale nanostructures during pupal development. Given that the papilionid honeycomb lattice extends into the lumen of the scale cells, any putative role of the SER in honeycomb morphogenesis should also be investigated. It would be of interest to follow the development of pupal wing scales using tissue clearing techniques or attempt a more finely resolved developmental time series to capture the full complexity of molecular and cytoskeletal dynamics. Comparatively understanding the morphogenesis of hierarchical nanostructures across biodiversity could inspire facile, biomimetic routes to synthesizing hierarchically structured materials for technological applications, given current challenges in the engineering of complex multi-functional mesophases (Kolle et al., 2010 [↗](#); Pokroy et al., 2009 [↗](#); Potyrailo et al., 2007 [↗](#); Siddique et al., 2017 [↗](#)).

Materials and Methods

Experimental design

To understand the process of scale nanostructure formation, we performed time-resolved imaging on developing pupal scales using specific biomarkers conjugated with fluorophores. Since cellular membranes and cytoskeletal elements have been identified as key components driving scale cell development, we used biomarkers targeting F-actin, cuticle (chitin), plasma membrane and Arp2/3 complex. We used 3D structured illumination microscope (3D-SIM) and lattice SIM to try and resolve structures beyond the diffraction limit. We would have preferred to present all confocal and SIM data solely on a single patch, for instance, the green dorsal forewing cover scales of pupal *P. eurimedes*. However, for time points where scales were highly crumpled or folded, we addressed this gap with data from black areas adjacent to the green patch, or from homologous yellow dorsal forewing patch on females. The overall consistency of the results validates our approach.

Dissection of pupae and tissue preparation

Pupae were purchased in multiple batches from Marl Insect and Butterfly Culture (Philippines), Stratford-upon-Avon Butterfly Farm (UK), and Mariposario del Bosque Nuevo (Costa Rica) between June 2017 - April 2021. Precise pupation dates were only available for *Papilio polytes* and one batch of *P. eurimedes* pupae. For *Parides* batches without information on the pupation dates, we were able to estimate the rough pupation stage (indicated by ~, e.g., Figs. 2A'''-C''' [↗](#), and 4) by morphological comparisons to this reference *P. eurimedes* batch. Sex of each pupa was determined by examining markings on the ventral segments. Wings from each pupa were dissected in cold Phosphate Buffer Saline (PBS) and immediately fixed in 4% PEM-PFA at room temperature for 15 mins. Following washes in PBS, wings were placed in blocking buffer (0.5% NP-40) at 4°C overnight

prior to staining. The average duration from pupation to emergence is 10 days for *P. polytes* and 21 days for *P. eurimedes*. We report the percentage of development in our time series for ease of comparison between species with different developmental rates.

Developmental time series with Wheat

Germ agglutinin, phalloidin and anti-Arp2

Wheat Germ Agglutinin (WGA) was previously used to visualize butterfly scale cell growth and is thought to initially stain plasma membrane before switching to chitin at later developmental stages (Dinwiddie et al., 2014 [DOI](#)). Phalloidin is a standard method to visualize F-actin. For AF-555 WGA and AF-647 phalloidin double-staining, pupal wings were incubated in 1:200 dilution of Alexa Fluor 555-conjugated WGA (Invitrogen W32464) and 1:40 dilution of Alexa Fluor 647-conjugated phalloidin (Invitrogen A22287) for an hour at room temperature. For FITC WGA and TRITC phalloidin double staining, *P. palinurus* pupal wings were incubated in 1:100 dilution of FITC WGA (EY Labs F-2101-5) and 1:100 dilution of TRITC phalloidin (Sigma P1951) for an hour at room temperature. Arp2 is the ATP-binding component of the actin Arp2/3 complex, which functions as an actin nucleator in branched actin networks (Goley and Welch, 2006 [DOI](#); Smith et al., 2013 [DOI](#)). After blocking, pupal wings were incubated with a 1:500 dilution of rabbit anti-Arp2 (Abcam ab47654; pblast search revealed Uniprot #P61160, Human Arp2 has 82% sequence similarity to XP_013178655.1, *Papilio xuthus* Arp2) at 4°C overnight. After washing, the wings were incubated in buffer with a 1:300 dilution of Alexa Fluor 594 Goat anti Rabbit secondary antibody (Abcam ab150088) for an hour at room temperature.

Time series with CellMask Plasma Membrane stain

As the CellMask plasma membrane stain does not survive permeabilization, dissected pupal wings were immediately stained with a 1:300 dilution of CellMask Deep Red (Invitrogen C10046) for 10 mins. After removing the staining solution, the wings were fixed in 4% PEM-PFA at room temperature for 15 mins. Following washes in PBS, pupal wings were stained with a 1:200 dilution of Alexa Fluor 555-conjugated WGA (Invitrogen W32464) for an hour at room temperature. In order to prevent permeabilization, the buffers for Cellmask stains did not contain any detergents (e.g. Triton).

Arp2/3 inhibition with CK-666

CK-666 was purchased from Sigma Aldrich (SML0006) and the stock solution was dissolved in DMSO. For pupal injections, CK-666 was diluted in 1x Grace's insect medium to final concentration of 100µM. Two to three *P. polytes* pupae were injected with CK-666 at 40%, 50%, 60%, 70%, and 80% development. Around 8-10µL of CK-666 is injected into wing pouches using a glass needle. Control pupae were injected with equal volume of DMSO diluted in Grace's insect medium. All injected pupae were dissected at 80% development and stained for immunofluorescence. Pupa injected on 80% development were allowed to develop for at least two hours before dissection.

Negative controls

For experiments with non-fluorophore-conjugated primary antibodies (Arp2 staining), we labelled a separate wing from the same individual with the secondary antibody only. This is to determine if the observed fluorescence signal is due to non-specific binding of secondary antibodies. After blocking, pupal wings were incubated without primary antibodies (only buffer) at 4°C overnight. After washing, the wings were incubated with a 1:300 dilution of Alexa Fluor 594 Goat anti Rabbit secondary antibody (Abcam ab150088) for an hour at room temperature. Negative controls were imaged using the same settings (laser power, gain, etc.) as the anti-Arp2 antibody-stained test samples.

Image acquisition, data processing

Following washes, the wings were mounted on glass slides in Prolong Gold antifade (Life Technologies P36930), covered with #1 thickness coverslips and sealed with nail polish. Confocal images were acquired using Olympus FV3000 (60x), Nikon A1R (100x) and Leica SP8 (100x). 3D-SIM was performed on a DeltaVision OMX and lattice SIM using Zeiss Elyra 7. Confocal data acquired with Olympus FV3000 and Nikon A1RSIM data were deconvolved using default settings in Huygens Professional v20.04. Confocal data acquired with Leica SP8 were deconvolved using default settings in Leica's Lightning deconvolution software during acquisition. All images were examined and processed using Bitplane Imaris Viewer 9.5.1. Movies were generated using Bitplane Imaris and edited using Shotcut v19.07.15 (Meltysch, LLC)

Critical point drying (CPD)

Wings from each pupa were dissected in cold PBS and immediately fixed in 4% PEM-PFA at room temperature for 15 mins. Following washes in PBS, wings were subjected to serial dehydration in 25%, 50%, 70%, 90% and absolute ethanol for 10 minutes each. The specimens were then transferred to the sample holders and dried using Leica EM CPD300 Automated Critical Point Dryer with the following settings: (CO₂ influx) Medium speed, 120s delay; (Exchange process) Speed 5, 14 cycles; Medium heating speed; and Medium gas out speed. It should be noted that direct air drying of soft samples *e.g.* pupal wing scales is not recommended, as water surface tension forces will result in significant distortion of specimen structure (Bray et al., 1993 [\[1\]](#)).

Conventional and focused ion beam (FIB) scanning electron microscopy (SEM)

Adult scales were individually picked with a needle and placed on carbon tape. Mounted scales were fractured using a razor-blade to obtain cross-sectional views. All samples were sputter-coated with platinum or gold to increase conductivity and reduce charging. Samples were imaged using JEOL JSM 6010LV Scanning Electron Microscope at 15-20kV.

For focused ion beam (FIB) milling, samples were prepared by sputter-coating with platinum to increase conductivity. The sectioned scale shown in **Fig. 1C** [\[1\]](#) is milled using a gallium ion beam on a FEI Versa 3D with the following settings: beam voltage - 8kV, beam current - 12pA at a 52° tilt. Image acquisition was performed in the same equipment with the following settings: beam voltage - 5kV, beam current - 13pA.

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Author Contributions

V.S. and K.S. designed the study. K.S. performed the experiments. Both authors analyzed the results and wrote the manuscript.

Competing interests

Authors declare no competing interests.

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Editors

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Reviewer #1 (Public Review):

This article is interested in how butterfly, or more precisely, butterfly wing scale precursor cells, each make precisely patterned ultrastructures made of chitin.

To do this, the authors sought to use the butterfly *Parides eurimedes*, a papilionid swallowtail, that carries interesting, unusual structures made of 1) vertical ridges, that lack a typical layered stacking arrangement; and 2) deep honeycomb-like pores (rather than. These two features make the organism chosen a good point of comparison with previous studies, including classic papers that relied on electronic microscopy (SEM/TEM), and more recent confocal microscopy studies.

The article shows good microscopy data, including detailed, dense developmental series of staining in the *Parides eurimedes* model. The mix of cell membrane staining, chitin precursor, and F-actin staining is well utilized and appropriately documented with the help of 3D-SIM, a microscopy technique considered to provide super-resolution (here needed to visualize sub-cellular processes).

The key message from this article is that F-actin filaments are later repurposed, in papilionid butterflies, to finish the patterning of the inter-ridge space, elaborating new structures (this was not observed so far in other studies and organisms). The model proposed in Figure 6 summarized these findings well, with F-actin reshaping itself into a tulip that likely pulls down a chitin disk to form honeycombs. These interpretations of the microscopy data are interesting and novel.

There are two other points of interest, that deserve future investigation:

1. The authors performed immunolocalizations of Arp2 and pharmacological inhibitions of Arp2/3, and found some possible effect on honeycomb lattice development. The inter-ridge region of the butterfly *Papilio polytes*, which lacks these structures, did not seem to be affected by drug treatments. Effects were time-dependent, which makes sense. These data provide circumstantial evidence that Arp2/3 is involved in the late role of F-actin formation or re-organisation.
2. The authors perform a comparative study in additional papilionids (Fig. 6 in particular). I find these data to be quite limited without a dense sampling, but they are nonetheless interesting and support a second-phase role of F-actin re-organisation.

The article is dense, well produced and succinctly written. I believe this is an interesting and insightful study on a complex process of cell biology, that inspires us to look at basic phenomena in a broader set of organisms.

Reviewer #2 (Public Review):

The manuscript by Seah and Saranathan investigates the cell-based growth mechanism of so called honeycomb-structures in the upper lamina of papilionid wing scales by investigating a number of different species. The authors chose *Parides eurimedes* as a focus species with the developmental pathway of five other papilionid as a comparative backup. Through state-of-the-art microscopy images of different developmental steps, the authors find that the intricate f-actin filaments reorganise, support cuticular discs that template the air holes that form the honeycomb lattice.

The revised manuscript is well written and easy to follow, yet based on a somewhat limited sample size for their focus species, limiting attempts to suppress expression and alter structure shape. I have no further comments.

Author Response

The following is the authors' response to the original reviews.

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Reviewer #2 (Public Review):

*The manuscript by Seah and Saranathan investigates the cell-based growth mechanism of so called honeycomb-structures in the upper lamina of papilionid wing scales by investigating a number of different species. The authors chose *Parides eurimedes* as a focus species with the developmental pathway of five other papilionid as a comparative backup. Through state-of-the-art microscopy images of different developmental steps, the author find that the intricate f-actin filaments reorganise, support cuticular discs that template the air holes that form the honeycomb lattice. The manuscript is well written and easy to follow, yet based on a somewhat limited sample size for their focus species, limiting attempts to suppress expression and alter structure shape.*

The fact that the authors find a novel reorganisation mechanism is exciting and warrants further research, e.g. into the formation of other microscale features or smaller scale structures (e.g. the mentioned gyroid networks).

We thank the reviewer for the positive appraisal.

The authors place their results in the discussion in the light of current literature (although the references could be expanded further to include the breadth of the field). However, the mechanistic explanation completely ignores the mechanical properties of the membranes as an origin of some of the observed phenomena (see McDougal's work for example) and places the occurrence of some features into Turing patterns and Ostwald ripening, which I find somewhat unlikely and I suggest that the authors discover this aspects further in the discussion.

We thank the reviewer for these suggestions. We have added more references from the current literature to more accurately reflecting the breadth of the field. McDougal et al. 2021. discuss the nature of biomechanical forces (differential growth and buckling) on the membrane and deposited cuticle shaping the formation of longitudinal ridges. However, here it is the invagination of the plasma membrane bearing the deposited cuticle that is our main concern. Nevertheless, we agree future studies should indeed consider the mechanical properties of the membranes, in addition, to explain some of the observed features. We have clarified this in our discussion.

I have little concerns regarding the experimental approach beyond the somewhat limited sample size. One thing the authors should more clearly mention are the pupation periods for all investigated species as only the periods for two species are named.

Yes, unfortunately, we were only able to obtain pupae with pupation dates for two species. We have clarified this point in the methods.

Reviewer #1 (Recommendations For The Authors):

Suggestion for improvement.

I recommend adopting a magenta/green (or orange/azure) color scheme to make the figures accessible to most color vision types. This does not require re-doing the figure and could be processed on the rendered JPG/TIF figures with the following procedure :

1. open the rendered figures in Photoshop in RGB mode

1. go to Channel Mixer

1. Select Output Channel : Blue

1. set Blue 100%-->0% and Red 0-->100%

This will change Red to Magenta without affecting luminosity.

Similar solutions should be available in other software including GIMP.

Of note this is a late fix and ideally, color encoding could be done upstream in the microscopy file extraction software (e.g. Fiji), but I do not think this heavier solution is needed here.

We thank the reviewer for this suggestion. In order to be more inclusive, we have redone the figures and videos in a yellow+magenta color scheme.

Reviewer #2 (Recommendations For The Authors):

References: Some literature is missing that could be considered by the authors e.g.

<https://doi.org/10.1098/rstb.2020.0505> <https://doi.org/10.1101/2023.06.01.542791>

<https://doi.org/10.1098/rsfs.2011.0082> <https://doi.org/10.1557/mrs.2019.21>

<https://iopscience.iop.org/article/10.1088/2040-8986/aaff39/meta> <https://doi.org/10.1364/OE.20.008877>

We have added more references as suggested.

Placing the captions next to the figures, particularly in the SI will help accessibility.

We agree. We believe this would be done during article production.

113: chiefly?

We have replaced 'chiefly' with 'focusing mainly on'.

160: how do you know the scales are more sclerotized already? Just because it's later in development?

Yes, that is what we are alluding to here. We have made edits to clarify this sentence.

186: Specify sample size.

We have specified the sample size '(N = 15)' here.

309: Multilayered cover scales would be more accurate.

Thanks for the suggestion. We have changed ‘structurally-colored cover scales’ to ‘multilayered cover scales’ as suggested.

| *Please check the literature list again for accurate references.*

Thanks for the suggestion. We have gone through the references and fixed any missing information.