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Structure Formation in Butterfly Scales: Interplay of Genetic Control, Mechanical Instabilities, and Dynamic Material Properties

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ABSTRACT

Butterfly scales contribute to a butterfly's vibrant coloration and play crucial roles in functions such as thermoregulation, water repellency, and aerodynamics. However, the underlying mechanisms that drive scale structure formation in vivo are not well understood. In this perspective, we propose that mechanical instabilities are central to the morphogenesis of scales and can lead to the observed wide variety of scale morphologies in adult butterflies. We specifically focus on the interplay between a growing soft compartment formed by the plasma membrane and an epicuticular envelope, the constraints imposed on this compartment by the actin cytoskeleton, and the spatio-temporally heterogeneous sclerotization of the cuticle precursors. We discuss hypotheses on how intracellular processes control the composition of the cuticle precursor secreted into soft compartments and how mechanical instabilities may lead to the morphological diversity of ridges, lamellae, and other scale structures. Putting forward a set of hypotheses about the fundamental mechanical processes that enable the secretion of non-living functional biological matter, we aim to inspire novel fabrication approaches in material science and engineering.

1 | Introduction

The colored patterns on the body and the wings of butterflies are due to scales, dried remnants of single epithelial cells, homologous to sensory hairs [1], that exhibit a remarkable structural diversity. Scale development is an excellent model for deciphering biological structure formation. It involves the secretion of non-living functional structures, revealing the underlying physical and chemical mechanisms, the key cellular and molecular components, and process parameters involved. Butterfly scales are among nature's structurally most diverse materials [2–7] featuring desirable optical [2–4, 7–9], thermal [10–12], chemical [13], and aerodynamic properties [14]. Each butterfly scale is produced by just a single cell [15, 16], with thousands of cells forming structurally near-identical copies possessing modular functionality. In each butterfly scale, roughly 100 µm long, 40 µm wide, and a few microns thick, multiple patterns at various submicron length scales are hierarchically integrated. On the upper side of the scale, periodically spaced ridges (about 500 nm to 2 µm apart) are decorated with regular lamellae (~100 nm

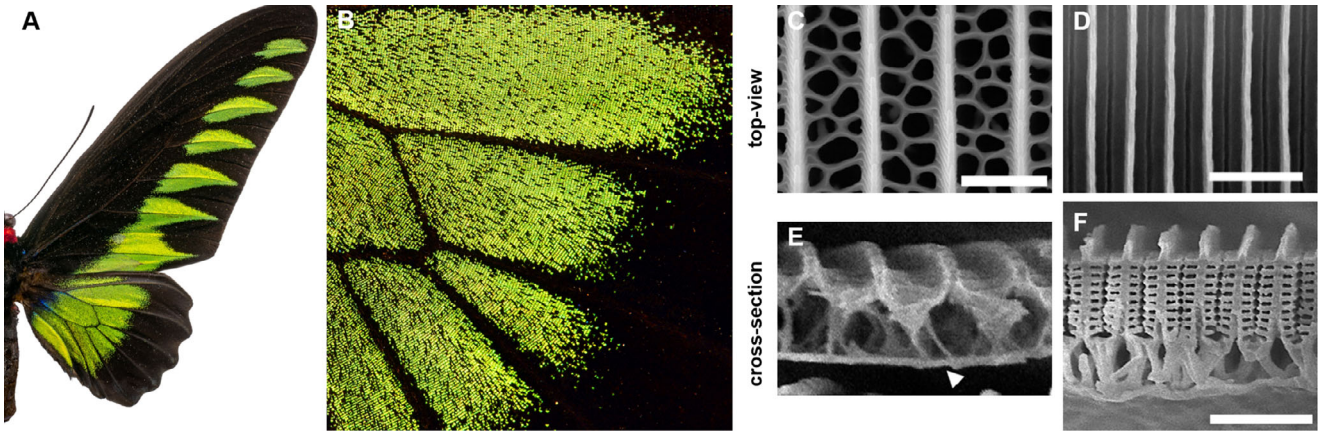

FIGURE 1 | Structuring of a butterfly at different length scales, the example of *Trogonoptera brookiana*. (A) This butterfly features bright green wing patches on a dark black wing. (B) Zoom-in on the hind wing shows scales and the wing veins. (C–F) SEM images of (C,E) black and (D,F) green-colored scales, (C,D) in top-view, (E,F) in cross-sectional view. (A,B) from the public domain (Adobe Stock, #298544565 and #105838854, (C–F) reproduced from ref. [27] under a CC-BY license. Scale bars: (C–F) 2 µm.

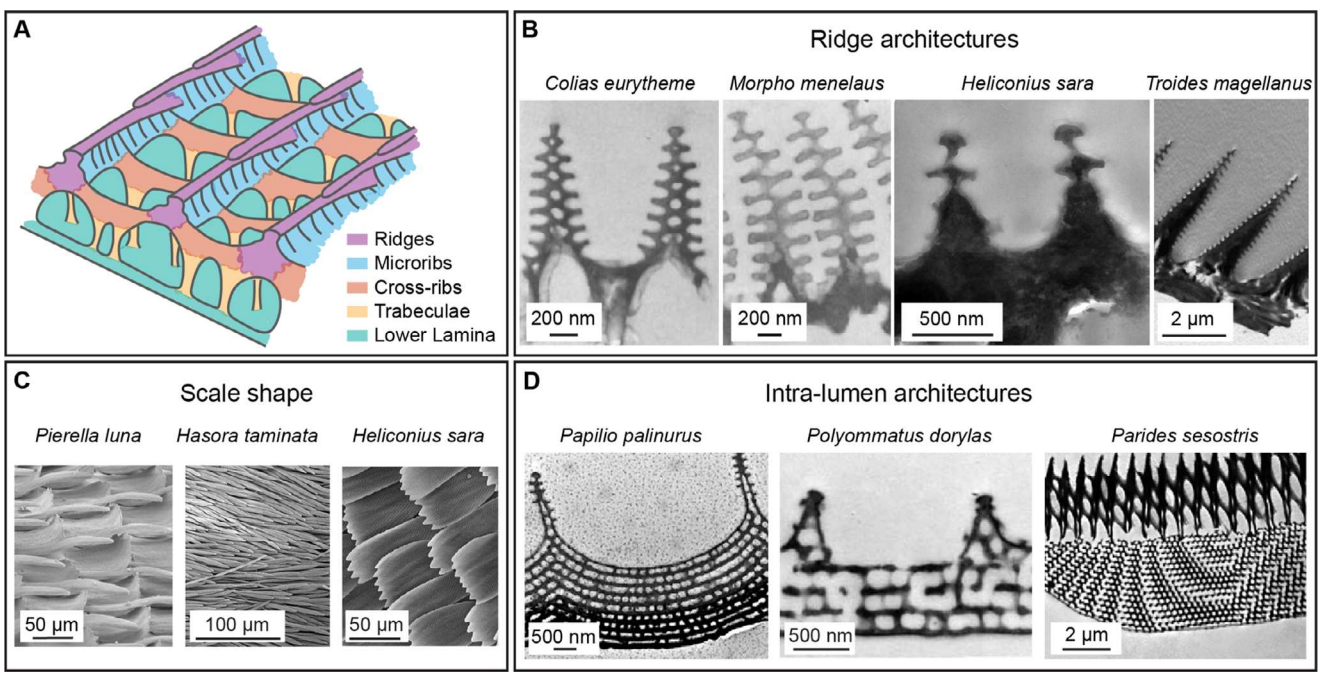

FIGURE 2 | Different scale variations in mature butterfly wing scales. (A) The generic structure of a butterfly wing scale (as in ref. [15]). (B) Transmission electron micrographs showing variations of wing scale ridge architectures from different butterfly species. Reprinted with permission from ref. [28] Copyright 1974 Wiley, and from ref. [29]. Copyright 2008, American Physical Society. (C) Scanning electron micrographs showing variations of wing scale shape. Reprinted with permission from refs. [30, 31], under a CC-BY license, and from ref. [32], Copyright 2014 National Academy of Sciences. (D) Transmission electron micrographs showing variations in intra-lumen structures from different butterfly species. Reproduced with permission from ref. [33], Copyright 2006 Company of Biologists, and ref. [34], Copyright 2012 the Royal Society.

thick) and linked by cross-ribs (about 200 nm to 1 µm apart; see Figures 1 and 2 for details) [15, 16]. Intra-scale structures can include pigment-filled beads, lamellar architectures, and gyroid networks with periodicities of a few 100 nm [17–25]. The lower lamina of the scale is a simple thin film, whose optical properties are often matched to those of the overlying structures [26]. Butterflies are excellent experimental models that can be reared in the lab and manipulated genetically, allowing detailed investigations into the formation of these structures.

The diversity in scale morphology—from simple flattened forms to complex three-dimensional architectures—is a testament to the intricate biological processes governing their development. Previous research has illuminated various aspects of scale

formation, such as the role of genetic factors in determining scale identity [35–37] and the biochemical pathways controlling pigmentation [38, 39]. However, the mechanical and cellular processes that lead to the diverse structural outcomes are less understood; only recently, research has moved toward time-dependent and in vivo investigations [17, 30, 40–42].

Recent work has found that the actin cytoskeleton, a network of filamentous proteins within the developing scale cell, is important for butterfly scale cell morphogenesis due to its role in defining scale shape and scale cell membrane configuration throughout critical stages of scale development [30, 41–43]. The secretion of cuticle precursors and their subsequent sclerotization—a hardening process involving water removal and chemical cross-linking of proteins and chitin, which has been hypothesized to involve mechanical instabilities [28], ultimately results in the formation of the rigid functional structures found in mature scales.

In this perspective, we integrate biological and mechanical hypotheses to propose a comprehensive overview of the phenomena and processes driving scale structure formation. We suggest that the interaction of the sclerotizing cuticle precursor and its soft compartment, which is formed by the plasma membrane and an epicuticular envelope, subject to constraints by the actin cytoskeleton, leads to mechanical instabilities that drive the formation of diverse scale structures. By exploring this hypothesis from a process point-of-view, focused on what the cell controls in its interior and how that affects the instabilities playing out in its exterior assembly compartment, we aim to offer new insights into the fundamental principles of biological structure secretion, and identify critical future research directions for broadly understanding biological secretion of functional materials, highlighting potential avenues for bio-inspired manufacturing approaches.

2 | Diversity of Functional Nanostructures in Butterfly Wing Scales

Butterfly scales exhibit a wealth of functional structural features, found on the scale surface, within the scale lumen [2, 15, 44], and even the lower lamina alone is a functional surface [26, 45–47]. These features include the spectrally-selective reflectors formed by regular lamella on scale ridges, as displayed by iridescent blue *Morpho* butterflies [3, 48–50]. Similar implementations of ridge-based lamellar photonic architectures are employed by many different species to reflect light in different spectral ranges, from the UV to the infrared [6]. In addition, many butterfly species form regular cuticle structures, including multilayers and gyroids within their lumen, or scattering materials such as pterin-based granules [24]. This structural diversity is present both within and between species, with most species exhibiting multiple different scale types, often in distinct color patches across the wing (see Figure 1 for an example of structuring on a single butterfly, and Figure 2 for an overview of different scale ultrastructure types across butterfly wing scales) [6].

We hypothesize that all this structural diversity results from a few physical and chemical mechanisms that the cell regulates through genetic control of the cytoskeleton, the soft compartment formed between an epicuticular envelope and plasma membrane, and the location, timing, and composition of cuticle precursor secretion.

3 | How Scale Growth Progresses—A Phenomenological Picture

Understanding scale morphogenesis requires examining the spatio-temporal occurrence and interplay of several processes. Scale development involves an interwoven sequence of cytoskeletal remodeling, membrane reshaping, material secretion, and material transformation.

Several different attempts have been made to define a framework of specific stages of structure formation with varying focus on developmental steps, distinct processes, and emergence of specific attributes [40, 51, 52].

Here, we refrain from trying to define specific stages but rather choose to discuss and categorize a series of phenomenologically observed events in scale formation. The focus for our conceptual framing and categorization is the utility of any phenomenologically observed cell configuration for the cell's overarching task of generating a set of defined cuticular architectures.

This allows for a simple categorization of events (Figure 3): scale-forming cells have to first be positioned in the wing epithelium and differentiate to specialize for the tasks involved in forming a scale. Each scale-forming cell then has to shape its geometry, cytoskeleton, and membranes to define their secretion compartments. Then the cell secretes its cuticle precursors into these compartments to form specific sets of the ultimately present scale features, such as ridges, cross-ribs, gyroids, or trabeculae. Each distinct scale feature requires different types of compartments; consequently, the cell undergoes several reconfigurations of its cytoskeleton and membranes to condition its secretion compartments prior to the subsequent secretion of another feature. Once the cuticle precursor for all features has been secreted and progressed sufficiently in its solidification, all that is left for the cell to do is the clean-up phase, where it retracts from its creation and undergoes apoptosis.

Although in the following we present and discuss these steps sequentially, they do not necessarily unfold all in a strictly linear fashion one-after-the-other. Instead, multiple processes may proceed concurrently and play out in parallel with significant variations in their onset, duration, and degree of overlap across individual scales and organisms, even of the same species. This variability underscores that the sequence of events presented here serves as a conceptual scaffold. In the description below, we refer to developmental timing as a percentage of pupal development. Unless otherwise stated, 100% of development refers to the time between pupation and adult eclosion, with percentages indicating the relative progression through the pupal stage.

In the description below, we emphasize the scale cells' changing geometrical attributes and the characteristics of the most important components involved in structure formation: the actin cytoskeleton, the plasma membrane, the epicuticular envelope,

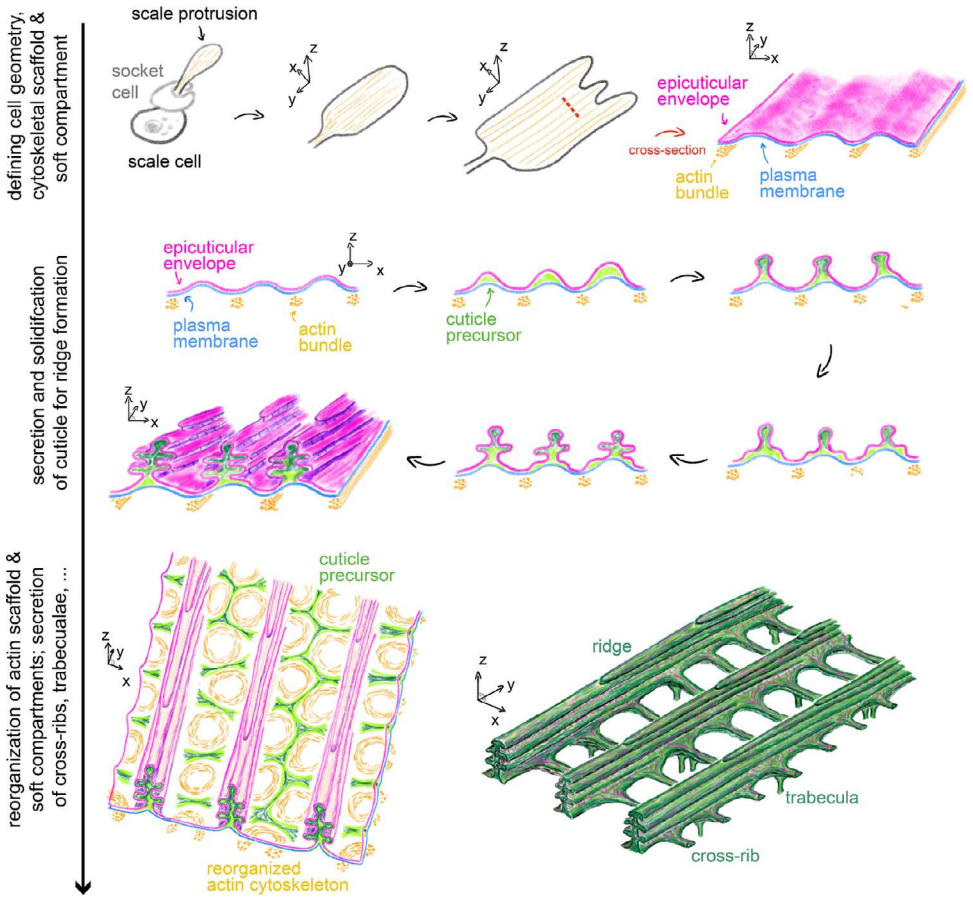

FIGURE 3 | Progression of scale formation, with focus on scale ridge and cross-rib architecture. Initially, the scale-forming cell defines its overall geometry, configures its cytoskeleton, and arranges the plasma membrane and outer epicuticular envelope to form a soft compartment for the cuticle precursor. The cuticle precursor is secreted and solidifies within this compartment. Once the ridge cuticle has sufficient mechanical stability, the cell reorganizes its actin skeleton to generate different constraints on the plasma membrane. Following this, cross-ribs form, and as secretion progresses, trabeculae are generated coinciding with the cell retracting its cytoplasm. Actin cytoskeletal structures are drawn in yellow and the epicuticular envelope in pink. Fresh, fluid cuticle precursor is shown in light green, and solidifying precursor in darker green.

and the cuticle precursor (details to these terms can be found in the glossary). These components interact mechanically and biochemically to define the scale's intricate architectures that are visible in the mature butterflies as presented in Figure 2.

A categorization focused on the utility of specific observable cell configurations for the cell's task to form a scale, rather than on species-specific developmental stages and timing, should be broadly applicable across Lepidoptera and potentially other insects with homologous cuticular structures. Where relevant, variations in timing or morphology are noted and discussed in more detail in the following sections.

3.1 | Positioning of Scale Precursor Cells and Differentiation Into Scale and Socket Cells

Early in the development of the pupal wing epithelium, scale precursor cells assume positions in ordered rows and columns [40]. This is a multicellular process involving the arrangement and coordination of multiple cells on the wing epithelium to ready scale-forming cells for their task. Each precursor cell then divides into two cells at around 15% of development (relative to the interval between pupation and adult eclosion) [43], one forming the socket cell, which surrounds the base of the scale, and the other becoming the scale cell.

3.2 | Defining the Shape of Scale Protrusion, Organizing Actin Cytoskeleton, and Secretion of Epicuticular Envelope to Prepare the Cell's Secretion Environment

Scales develop as cytoplasmic projections from individual scale cells in the wing epithelium (Figure 4A). Between 20%–25% of pupal development the scale cell starts to grow a protrusion from the wing surface, confined by the socket cell [30, 43]. Initially, the cellular protrusion that forms the scale is fairly cylindrical in cross-section and elongates in length [43], similar to a bristle. The scale then begins to widen as well as continuing to grow in length but not in height, becoming elliptical in cross-section, at this point entering a distinct phase of development that clearly differentiates it from a bristle.

Actin filaments that are present in the scale protrusion reorganize from a diffuse network into longitudinal bundles that run

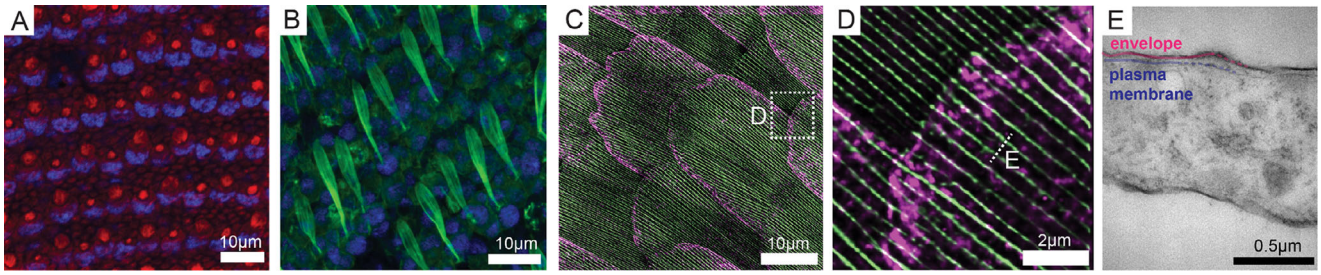

FIGURE 4 | Shaping up scale geometry, cytoskeleton, and epicuticular envelope. (A) The scales are just beginning to bud from the wing epithelium. Microtubules, inside the emerging scales, are shown in red (Anti-alpha Tubulin immunostaining). Cell nuclei are shown in blue (DAPI staining) (B) The scales have elongated; longitudinal actin bundles are shown in green (phalloidin staining). (C,D) Actin (green, phalloidin) has assembled into periodically arranged bundles underneath the plasma membrane. Some chitin (magenta, chitin-binding-domain probe) is just beginning to be deposited at the edge of the scale. (D magnifies the area marked in C). (E) A TEM cross-section of a scale. The epicuticular envelope is the dark layer seen on the outside of the cell (highlighted in pink). The cell membrane can be seen just below this envelope (highlighted in blue). Fluorescent confocal microscopy images (A,B) and tauSTED super-resolution micrographs (C,D) reproduced with permission from ref. [30], under a CC-BY license. All images are acquired from *Heliconius sara* but reflect similar observations in other species.

along the forming scale and are likely supporting its elongation (Figure 4B) [43]. Outside the cell membrane, a thin (~5–10 nm) layer, the epicuticular envelope (Figure 4E), is secreted. The early secretion of this outer layer is consistent with what is seen in the formation of other insect cuticles [53, 54]. This outer layer has previously been termed the "cuticulin" layer [28, 55, 56]. Its exact composition in butterflies is not known, but in other insect cuticles, it is thought to be composed of lipids and proteins [54, 55]. In the fruit fly, this envelope and the rest of the epicuticle, which thickens gradually through development, are thought to not contain chitin and to be composed of quinones, lipids, and several different proteins [53].

The dimensions of the forming scale have become established at around 40%–50% development. Actin filament bundles have fully formed and organized into periodically spaced longitudinal arrays, generally between about 0.5 to 2.5 μm apart (Figure 4C,D), that define the spatial limits of the developing scale ridges. These bundles create a periodic scaffold that associates closely with the plasma membrane and also appears to constrain the epicuticular envelope. Self-organization of actin into such highly ordered states is uncommon in other cellular contexts; while actin bundle formation has been studied in fruit flies [57, 58], the emergence of a periodic arrangement of actin bundles is not understood in the context of butterfly scale morphogenesis.

The epicuticular envelope defines a soft compartment in conjunction with the plasma membrane into which the secretion of cuticle precursor can now occur. However, the envelope has not yet finished growing [7, 59]. Undulations appear on the forming scale's upper surface and reshape due to growth and buckling of the cell membrane and the epicuticular envelope within the constraints imposed by the periodically spaced actin bundles [59]. In some scales, such undulations can also form on the scale underside [60], although for the majority of species, the lower scale lamina is ultimately fairly flat and usually does not exhibit ridges. The scale geometry does not change significantly anymore; however, in a number of species, scale "fingers" – undulations at the distal end of the scale – might still be forming.

3.3 | Secretion of Cuticle Precursor Into the Soft Compartment Between Epicuticular Envelope and Plasma Membrane, Progressing Sclerotization of Forming Cuticle

At roughly 50%–60% of pupal development, scale ridges begin to form in the locations where the epicuticular envelope and plasma membrane have buckled, between the actin bundles (Figure 5). These buckles of the epicuticular envelope and the plasma membrane appear to function as isolated soft compartments into which cuticle precursor is secreted. The envelope is initially compliant, accommodating changes in volume and shape as cuticle precursor material is deposited. The cuticle precursor is a complex mixture comprising the polysaccharide chitin, different cuticular proteins, water, and other small molecules [53, 61, 62], added in proportions that likely vary with location, time, and across species. As the precursor begins to mature, it undergoes sclerotization—a process involving cross-linking reactions that solidify the material. During sclerotization, water has to be expelled from the precursor back into the cell, leading to a spatially heterogeneous reduction in precursor volume and shrinking that is accompanied by the formation of lamellae, and later micro-ribs on the ridges.

3.4 | Reorganization of Actin Cytoskeleton in Preparation of Secreting Cross-Ribs, Trabeculae, and Other Cuticular Structures

Once the scale ridges have gained sufficient structural integrity, they appear to not require support by the actin bundles anymore (Figure 6). From around 60% of development, the actin cytoskeleton reorganizes multiple times. Initially, the large bundles break down, and a transient network first appears under the ridges [30, 43]. To some extent, this network appears to prefigure finer structures such as the microribs, which run down the side of the ridges [30], although further work is needed to determine if the actin is leading or following the formation of these finer features. The actin then forms periodic ring-like arrangements between the ridges. These ring-like actin structures have been observed in multiple species and appear to be important for the subsequent

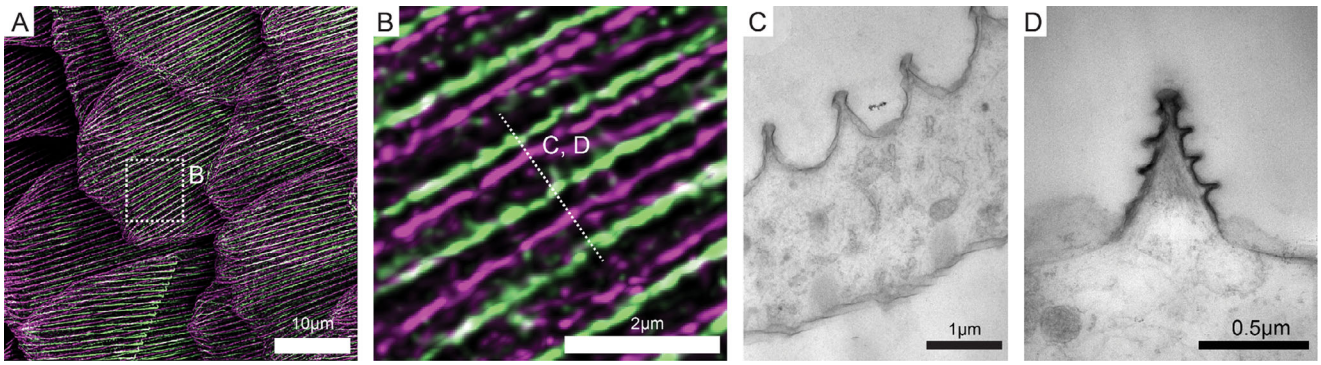

FIGURE 5 | Actin bundles constrain a soft compartment for ridge formation, cuticle secretion, and solidification. (A) Actin bundles (green, phalloidin) are periodically spaced and cuticle (magenta, chitin-binding-domain probe) is present in between, indicating ridge formation is in progress. (B) A magnified view of a scale in (A) clearly shows the alternating arrangement of actin and chitin. (C) TEM cross-section of a scale relatively early in ridge formation. The cell membrane and the epicuticular envelope form a soft compartment for the cuticle precursor that begins to assume a ridge-like morphology. (D) TEM cross-section of a single ridge at a later time point in ridge formation. All images are from *H. sara* but reflect similar observations in other species. TauSTED super-resolution images reproduced with permission from ref. [30], under a CC-BY license.

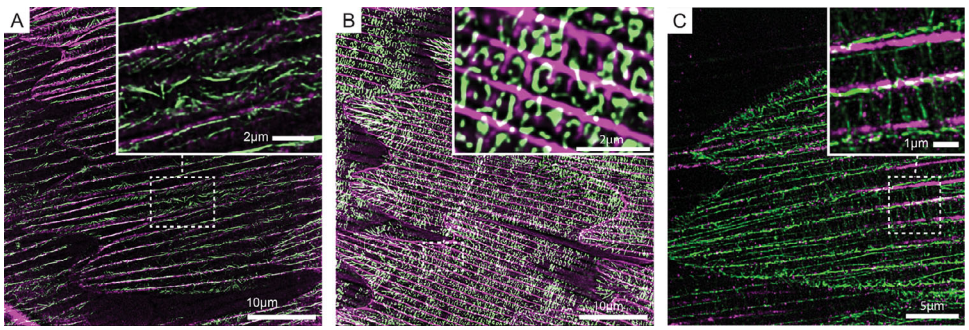

FIGURE 6 | Reorganization of the actin scaffold. (A) In *H. sara* scales, the actin (green, phalloidin) has rearranged under the scale ridges (shown by the presence of chitin, magenta), and the fine filaments appear somewhat like the microribs that decorate the sides of fully-formed scales. (B) Later in development, the actin is organized in rings between the scale ridges. There is evidence in this data that cross-ribs (magenta) are beginning to form between the actin rings (green). (C) Similar effects are observed in *Morpho helenor* scales, which also show actin underneath and linking between the ridges, illustrating the generality of these developmental stages across species. TauSTED super-resolution microscopy reproduced with permission from ref. [30], under a CC-BY license with actin (phalloidin) in green and chitin (chitin binding domain probe) in magenta. Inset boxes are zoom-ins of the dashed box regions.

formation of the cross-ribs, trabeculae, and the honeycomb lattice observed in some papilionid species [30, 41, 63].

3.5 | Secretion of Cross-Ribs, Honeycomb Lattices, Intra-Lumen Structures, and Trabeculae

The continuously evolving reorganization of the actin cytoskeleton provides new constraints on the plasma membrane and epicuticular envelope in the interstitial spaces between the ridges, defining the soft compartment necessary for the secretion and formation of cross-ribs, honeycomb lattices, and trabeculae.

It is also during this phase that structures that fill the scale lumen, such as gyroids and perforated multilayers, begin to form, although these are enabled by a different set of endomembrane-based soft compartments and driven by different processes [41, 64]. In papilionids, it has been shown that the intra-lumen structures form after the honeycomb lattice between the ridges, and in the gyroid-forming lycaenid species *Callophrys gryneus* it is clear that the ridges mature before the development of the gyroid [17, 64]. While further work is needed to uncover the details of intra-lumen structure formation, there is good evidence that gyroids form as a result of the complex folding of plasma membrane and intracellular smooth endoplasmic reticulum which templates the precursor cuticle deposition [18, 21, 64, 65]. In species that do not fill the scale lumen with gyroids, lamellae, or other nanoscale periodic architectures, trabeculae are forming as the scale cell retracts from its secreted cuticular architecture [15, 66].

3.6 | Pigment Deposition, Cell Retraction, and Finalization of Scale Structure

In the final phase of scale formation (>90% of development), ridge structures are fully developed. Pigment is either deposited within parts of the cuticular structures that are still forming or chemically converted within already formed cuticular components [38, 39, 63]. The cell cytoplasm completely withdraws from the formed cuticular exoskeleton, which likely contributes to some final shaping of cuticle structures, such as the trabeculae

[15, 66], before the cuticle proceeds to fully harden into its final form.

4 | How a Cell Patterns a Structured Scale: Hypotheses on Mechanisms, Process Parameters and Controls

The overarching assumption in this perspective is that the wide variety of scale morphologies found on butterfly scales – and in particular the various ridge structures – primarily arise from the interplay between genetically-controlled biochemical synthesis processes and mechanical phenomena (instabilities and minimization of potential energy) during scale formation. This forms the basis for establishing mechanistic hypotheses for scale structure formation valid across multiple species.

Our discussion of potential scale structure formation mechanisms focuses on the formation of the complex ridge and cross-rib structures found on the surface of the scale, as these are among the most prominent features contributing to scale diversity. Mechanisms that could drive the formation of intra-lumen nanostructures, including gyroids have previously been proposed and are discussed in greater detail in several publications elsewhere [17, 18, 20, 21, 65].

Here, we present and detail a set of hypotheses that outline our perspective on the interplay of potential processes and cellular components that enable the scale-forming cell to pattern cuticle ridges and cross-ribs on its exterior surface. These hypotheses address roles and organization of the intracellular cytoskeleton, the cell's exterior assembly compartments, and the nature of the solidifying cuticle precursors. We specifically hypothesize that the interaction between a growing soft compartment—created between the plasma membrane and the epicuticular envelope—and the spatially heterogeneous reduction in volume of the secreted sclerotizing cuticle precursor leads to hierarchical mechanical deformations, which give rise to the complex shapes of mature scale ridges. The actin cytoskeleton plays a crucial role in this process by providing structural constraints on the soft compartment in which the cuticle precursor matures. We hypothesize that the periodically spaced actin bundles constrain the plasma membrane and the epicuticular envelope. In addition, the actin bundles might also influence where the cuticle precursor is deposited. As the cuticle precursor undergoes sclerotization in the soft compartment between the plasma-membrane and the epicuticular envelope, we expect it to experience volume reduction due to water removal and cross-linking reactions. This volume change, constrained by the actin-defined boundaries of the soft compartment, results in mechanical stresses that drive deformation of the epicuticular envelope and the formation of ridge structures. This set of hypotheses emphasizes the importance of mechanical instabilities driven by biochemical processes within a soft assembly compartment, which in turn is constrained by a uniquely organized actin cytoskeleton.

4.1 | Organization of the Actin Cytoskeleton

Self-organization of actin into the highly ordered bundles found in scale-forming cells of Lepidoptera is uncommon in other cellular contexts. Instead, actin filaments often form either a network of crosslinked and entangled filaments, thick contractile cables called stress fibers, or long isolated bundles used for sensing (filopodia) [67, 68]. The formation of actin bundles, the underlying molecular principles, and the resulting morphologies have been studied in fruit fly bristles and in mosquito bristles and scales, involving several specific proteins that bind to actin [57, 58, 69–71]. Initial work suggests that at least one of the same proteins (Singed/Fascin) involved in fruitflies is also involved in bundle formation in butterflies, suggesting that the process may be quite similar [72]. The organization of actin bundles into a periodic longitudinal array underneath the plasma membrane and later into other structures underneath and between ridges is essential and likely the starting point for the formation of almost all scale structural features [30, 41, 43, 59, 72]. However, how this highly regimented periodic patterning is established and what controls its periodicity (which is very precise within a scale-type but varies between species and scale types) is not well understood. It is likely governed by intracellular processes, which the toolset of soft matter physics might help explain. We discuss below two relevant mechanisms that could lead to the periodic arrangement of actin filaments in different cellular contexts: membrane-mediated assembly of actin–membrane linker proteins and pattern formation via reaction-diffusion processes playing out directly in the actin cytoskeleton.

4.1.1 | Actin Organization Mediated by Membrane-Associated Proteins

In neuronal axons, actin is organized into periodically spaced rings along the axon circumference, with a regular spacing of 180 to 190 nm. Spectrin, a protein known to crosslink actin and to act as a molecular scaffold that connects the actin cytoskeleton to the plasma membrane, forms a periodic structure intercalated between two actin rings [73]. The periodicity of the axonal actin ring patterns is similar to the length of a spectrin tetramer, which could suggest that actin and spectrin self-assemble into a periodic structure, mediated by interactions with the plasma membrane. A similar process might play out in the organization of actin into periodically spaced bundles underneath the plasma-membrane of the scale-forming cell, although the periods between actin bundles are an order of magnitude higher (~1 μm) compared to the structures in axons.

4.1.2 | Reaction-Diffusion Mediated Pattern Formation

Another striking example of periodic actin organization can be found in *Drosophila* development, both in bristle and trachea formation [57, 74, 75]. In both instances, the periodic spacing between the actin bundles is on the order of 1 μm, similar to that seen in butterfly scales. Both developmental processes also involve the secretion of cuticle, much like during butterfly scale formation. It has been proposed that the periodic spacing of the actin bundles observed during trachea formation is governed by pattern formation [76] via reaction/diffusion/advection. Experiments and modeling suggest that the periodic arrangement results from the interplay between myosin contractility and actin turnover [74] while bundle orientation is the result of actin

assembly driven by stress-sensing formins [77]. At this moment, the physical and biochemical mechanisms that control the formation of periodic actin bundles in butterfly wings are unknown. However, the abundance of similarities between scale and trachea development, in particular regarding the actin–cuticle interplay and the similar spacing between bundles, suggests that reaction-diffusion mediated pattern formation is a solid hypothesis to test.

4.2 | Structuring of Developing Cuticle Precursor in Soft Extra-Cellular Compartments

To explain the formation of the intricate cuticle structures in scales, we have to uncover how the scale cell controls secreted materials in the extra-cellular environment. In the case of ridge structure formation, we specifically have to understand the dynamic mechanical interaction between the growing soft compartment (formed by plasma membrane and epicuticular envelope) and the sclerotizing, volume-changing cuticle precursor. During ridge formation, the cell secretes a malleable cuticle precursor consisting of proteins, chitin, and water through the plasma-membrane into this compartment. This compartment is periodically confined by a tight association of the regularly arranged actin bundles with the plasma membrane and the epicuticular envelope, localizing precursor accumulation between actin bundles. Once secreted through the plasma membrane, the precursor immediately starts to mature, while more precursor is secreted underneath. As the precursor undergoes volume reduction and hardens, it mechanically stresses and deforms the surrounding compartment. The presence of the actin cytoskeleton appears to impose a spatial constraint on the cell membrane and also the epicuticular envelope restricting the soft compartment's possible deformations. Such resistance can lead to mechanical instabilities, such as the buckling of the epicuticular envelope, collapsing the precursor material underneath into specific shapes. Taking into account the hardening of the cuticle precursor against the envelope, the formation of lamellae and micro-ribs could be explained by these buckling phenomena. The spatial distribution of these structures would be influenced by the initial organization of the actin cytoskeleton, the properties of the envelope, and the spatio-temporal progression of cuticle maturation. In this hypothetical framework, which emphasizes the mechanical interaction between the maturing secreted cuticle precursor and its growing soft compartment, only a small number of parameters are critical:

4.2.1 | Growth Rate of the Epicuticular Envelope and the Cuticle Secretion Rate

The ratio of envelope growth rate and cuticle secretion rate affects the evolving surface-to-volume ratio of the soft compartment and its cuticle content, which – if mismatched – is expected to lead to deformations of the epicuticular envelope. It is worth emphasizing that the envelope continues to grow as cuticle precursor is secreted into the soft compartment, as evidenced by ridge cross-sections obtained throughout ridge development [28]. It appears unlikely that the molecules needed to build the envelope are delivered by diffusion through the already secreted cuticle precursor, which is in the process of densifying and

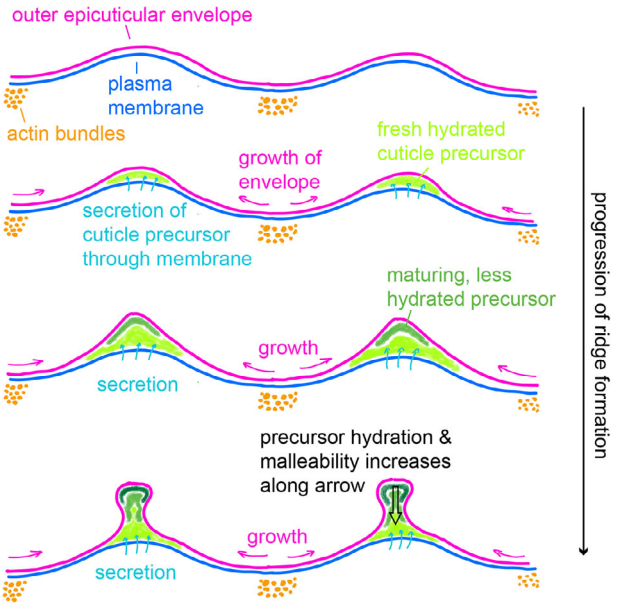

FIGURE 7 | As ridge formation progresses, the growth rate of the epicuticular envelope and the rate of cuticle precursor secretion are two important parameters. Cuticle precursor starts to sclerotize immediately upon secretion through the plasma-membrane while additional precursor is secreted underneath. This results in a systematic variation of the precursor material properties and its progression from a fluid to a solid from the top of a ridge to its bottom and periphery.

hardening. A more plausible hypothesis is that the epicuticular envelope grows in regions where it is more closely associated with the plasma membrane and where the cuticle precursor is not secreted, which is in the proximity of actin bundles. Cuticle precursor, by contrast, is secreted through the plasma membrane between the actin bundles. This hypothesis implies that the cuticle precursor and epicuticular envelope are sourced at different locations along the plasma membrane. We propose that the growth of the epicuticular envelope stems from a region close to the actin bundles, while the cuticle precursor moves from the plasma membrane out and up in the regions between bundles (Figure 7).

4.2.2 | Material Properties and Composition of the Cuticle Precursor

Viscosity of the cuticle precursor is likely a parameter that has an impact on its movement within the soft compartment and hence on the interaction with the enclosing growing epicuticular envelope. The viscosity, initial hydration, and degree of shrinkage upon sclerotization of the cuticle precursor are expected to be a function of the ratio of water, chitin, and cuticle proteins. Cuticle proteins with hydrophilic domains and hydrophobic chitin-binding domains could mediate interactions between water and the hydrophobic polysaccharide chitin. While chitin molecules might be directly synthesized by membrane-bound chitinases into the soft compartment, cuticle proteins are likely secreted by exocytosis of vesicles transported from cellular locations where proteins are synthesized and processed, such as the endoplasmic reticulum and the Golgi apparatus [78]. The hypothesis of vesicular exocytosis for at least some of the

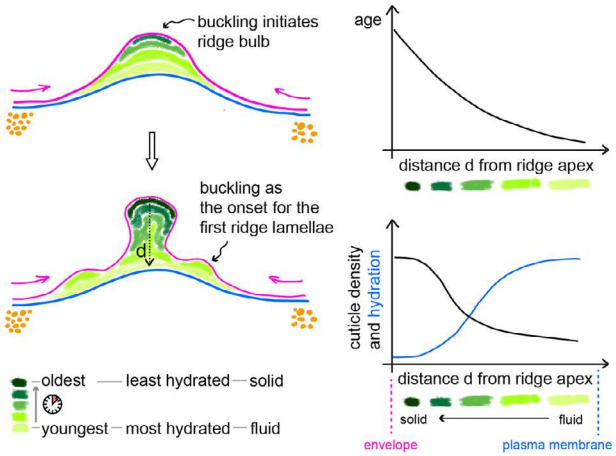

FIGURE 8 | Cuticle precursor is secreted sequentially; we hypothesize that the precursor starts to sclerotize as soon as it is secreted, which results in a systematic gradient of the precursor's mechanical properties and density in the soft compartment. The graphs on the right qualitatively sketch cuticle precursor age, density, and hydration as a function of distance d from the apex of the developing ridge.

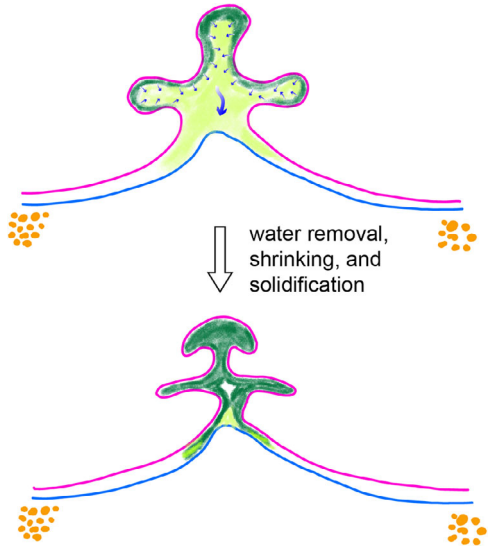

FIGURE 9 | Cuticle precursor sclerotization and the associated removal of water ("flow" of water shown by the blue arrows in the top drawing) leads to shrinking, solidification, and refinement of the initial buckled ridge morphology.

cuticle precursor ingredients is supported by visual evidence of an increase in plasma membrane area underneath the forming ridge [28]. Microtubules likely provide the transport pathway for such vesicles [78].

4.2.3 | Spatio-Temporal Dynamics of Cuticle Sclerotization

The spatio-temporal characteristics of sclerotization (cuticle precursor maturation) appear to be important for determining the structural outcome. Precursor material secreted earlier, adjacent to the epicuticular envelope, begins to harden sooner than material secreted later and located closer to the plasma membrane. We expect the older regions of the envelope to be associated with earlier secreted, more sclerotized cuticle precursor; consequently, older envelope regions around the ridge apex are expected to be stiffer than newer envelope regions located toward the ridge edges. In general, cuticle maturation together with the removal of water from the precursor (see below) are expected to lead to spatio-temporal variations in the mechanical properties of the forming cuticle affecting bending stiffness and hence configuration of the epicuticular envelope (the free top surface of the soft compartment). Spatial heterogeneity of the envelope's bending stiffness resulting from the age of the cuticle deposited against it (i.e., older cuticle had more time to mature and is therefore less malleable) would define a bending stiffness gradient along the envelope being highest in the apex of the forming ridge and decreasing toward the actin bundle constraints (Figure 8). That stiffness gradient would influence the field of mechanical stresses across the envelope and cuticle precursor and therefore affect the configurations that the envelope can assume to account for a difference in its growth rate and the cuticle secretion rate. As sclerotization progresses, the envelope may change in surface area and effective bending stiffness (cuticle hardened against it adds additional bending stiffness to the envelope's innate likely rather low bending stiffness). Initially flexible, the envelope thus becomes more rigid as it interacts with the hardening precursor. This imparts specific local conditions on the epicuticular envelope configuration as mechanical buckling instabilities arise, affecting the resulting ridge shape. Thus, the interplay between the changing properties of the epicuticular envelope and the sclerotizing precursor appears to be essential for the development of the final ridge morphology.

4.2.4 | The Role of Water During Cuticle Precursor Maturation

Water plays a dual role in the development of ridges. During the initial stages of precursor deposition, it acts as a transport solvent and plasticizer for the proteinaceous cuticle precursor (likely transported to the plasma membrane via vesicles and deposited via exocytosis). This ensures that the precursor is initially malleable and can deform in response to stresses in the interaction with the epicuticular envelope. We hypothesize that during sclerotization, the cross-linking of proteins, chitin, and other precursor ingredients decreases the miscibility, changing the phase equilibrium and enabling the expulsion of water back toward younger (less-cross-linked) precursor regions and ultimately back into the cell. This sets up a hydration gradient in the maturing cuticle pointing from the earlier secreted cuticle volumes near the epicuticular envelope back toward the later secreted volumes near the plasma membrane. Removal of water leads to a reduction in volume, exacerbating mismatches in surface-to-volume ratio, forcing further reconfiguration of the envelope (Figure 9). Additionally, the removal of water also alters the precursor's mechanical properties, increasing its stiffness and resistance to deformation, thereby constraining where and to what extent the envelope can reconfigure and respond to the surface-to-volume mismatch. The water's ability to move through the plasma membrane and epicuticular envelope is essential for these processes. Differences in water permeability of the

epicuticular envelope and membrane could, therefore, influence the rate and extent of sclerotization, affecting the mechanical stresses and resulting structures. We expect the envelope to show very low water permeability in line with published reports on the properties of epicuticular layers [79, 80].

4.2.5 | Spatio-Temporal Distribution of Precursor Ingredients

The cell influences the processes evolving on its exterior by controlling the location- and time-dependent composition of secreted epicuticular envelope and cuticle precursor materials. When, where, and in which proportions these materials are secreted determines how their mechanical interactions unfold and what structures result. Variations in the ratio of proteins to chitin in the mixture are expected to affect the precursor's hydration level, sclerotization kinetics, and mechanical properties. A precursor with higher protein content may retain more water, leading to greater volume reduction upon dehydration, whereas a higher chitin content may result in a stiffer, less deformable material. In addition, a vast array of different cuticle proteins is encoded in lepidopteran genomes and expressed in scale cells. Variations in the chemical properties of these, together with their relative abundances and timings of expression, will influence the properties of the precursor through development [81]. These compositional differences are expected to lead to variations in ridge size, spacing, and ridge morphology.

4.2.6 | Actin's Role in Controlling the Constraints on Soft Compartment and Precursor Secretion

The configuration of actin filaments into periodically spaced bundles determines the initial conditions for scale development. By attaching to and constraining the plasma membrane and likely also indirectly (via trans-membrane proteins) the epicuticular envelope, actin bundles influence where the cuticle precursor can be secreted, where the envelope grows, and how the soft compartment can deform to respond to the developing cuticle precursor. For example, areas with dense actin bundles may restrict precursor deposition, leading to variations in the thickness and composition of the cuticle across the scale. Moreover, the actin cytoskeleton is itself dynamic. After the initial formation of ridges, actin temporarily reorganizes underneath the ridges in a pattern similar to the micro-ribs observed on fully formed ridges. In some species, it then forms ring-like structures that influence subsequent stages of development, such as cross-rib formation. This reorganization highlights the importance of the temporal dynamics of the cytoskeletal constraints imposed on precursor assembly within the soft compartment.

4.3 | Genetic Control

In order to produce the consistent differences in scale morphology that are observed between species, and between different scale types within-species on the same wing, these must be under genetic control. The processes outlined above give some hint as to what these genetic "handles" might be. Genetic changes that alter proteins found in the plasma membrane, epicuticular envelope, or cuticle precursor could all alter the shape of the structures that are formed. Small differences in actin-binding proteins could influence the organization of actin, and differences in proteins that mediate interactions between actin, the plasma membrane, and the epicuticular envelope could be crucial for forming soft compartments of different sizes and constraints. These differences can act in a cell-autonomous way, only influencing the morphology of the cell they are expressed in, or a non-autonomous way, also influencing neighboring cells [82]. Differences in the proteins themselves may not be necessary, with differences in just the amount or timing of expression possibly being sufficient to alter the biochemical or biomechanical processes unfolding. It is worth remembering most butterfly species produce numerous different scale types on their wings, each with very different morphologies, all encoded by the same genome.

To date, work on the genetic basis of scale morphological differences has mostly identified transcription factors (Figure 10). For example, in sulfur butterflies (*Colias eurytheme/philodice*) the *bric-a-brac (Bab)* gene controls a switch between scales with UV reflecting scale nanostructures and those without [83]; *doublesex* controls structurally-based UV color in dogface butterflies (*Zerene cesonia*) [84]; and *optix* regulates structural color produced by thin-film interference in several species [36, 45, 85]. Transcription factors are regulatory genes that control the expression of other genes by binding to regulatory regions of the DNA sequence. Therefore, the identification of transcription factors as regulators of structural color formation, while interesting for telling us how butterflies control the regulatory networks that switch between different scale types, tells us little about the cellular processes directly shaping scale structure formation.

The reason that classical genetic approaches, identifying associations between genotype and phenotype, have so far largely identified transcription factors may be because these have focused on systems where there is a clear and discrete switch between one scale morphology and another. These switches will require concerted changes in multiple genes, making it likely that regulatory genes such as transcription factors are responsible. To identify the downstream actors controlling specific aspects of scale morphology, it may be informative to target systems where genetic differences do not cause a discrete switch but instead produce a range of more subtle and continuous variation in scale morphology. The *Heliconius* (longwing) butterflies provide such a system, with two species, *Heliconius erato* and *Heliconius melpomene*, showing continuous variation in structural color and scale morphology in genetic crosses between individuals with and without a structural blue color, suggesting the action of multiple genes [86, 88, 89]. Genomic and transcriptomic analysis and comparison of these populations have identified genomic regions and some genes within these [86], which could warrant further investigation.

Work on the molecular genetic control of butterfly scale structures is really only just starting, and is largely using knowledge based on fruit fly (*Drosophila*) bristle formation as a starting point. Interesting work by Adler [90, 91] has identified the gene *dusky-like* (*dyl*), encoding a Zona Pellucida (ZP) domain protein, as

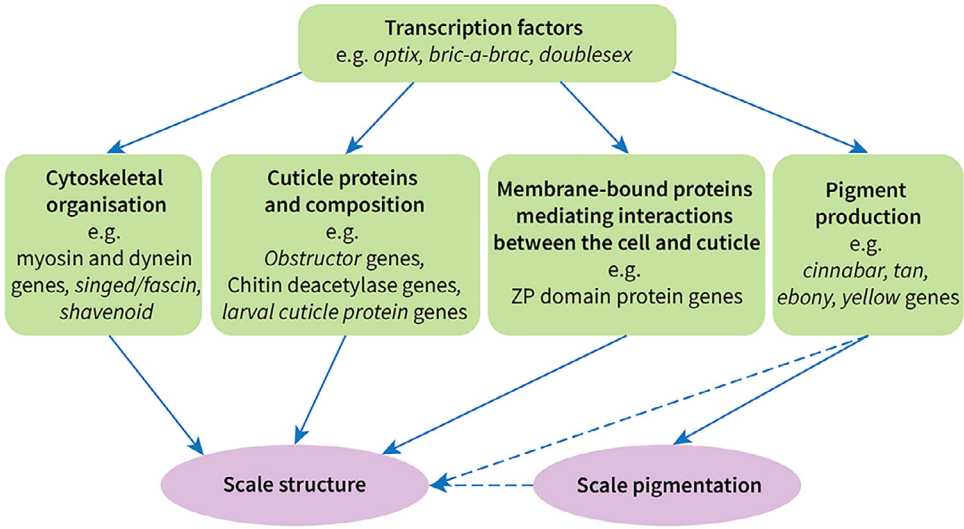

FIGURE 10 | Genetic control of scale structure. Transcription factors and other regulatory genes control downstream processes that determine scale structure formation including the composition and organization of the cytoskeleton (actin and microtubules) and the production, composition, and maturation of the cuticle. Examples of genes identified from genomic and transcriptomic studies are given [85–87]. Many of the identified transcription factors concurrently regulate pigmentation, and pigmentation genes, either directly or indirectly, can also influence scale structure [39].

mediating interactions between actin, the cell membrane, and chitin, possibly through localization of chitin synthase to the regions of the cell membrane where bristle ridges will form. This gene appears to be necessary for bristle ridge formation, making it a promising candidate to pursue further, given the similarities between fly bristles and butterfly scale ridges. This highlights that the scale structures are partially patterned through localization of specific proteins by the cytoskeleton. It also raises the alternative hypothesis that the cell's secretory elements and, therefore, scale structures may be directly patterned by the cell's molecular machinery, for example, following the patterning of actin, rather than biomechanical processes unfolding outside the cell.

5 | Approaches to Evaluate the Presented Hypotheses

The hypotheses presented above aim to paint a coherent picture between critical mechanisms and cellular components that ultimately define the processes of cellular secretion of functional materials. Their evaluation requires visualization and quantitative metrology of key cellular components accompanied by modeling, measurement, and manipulation of their presence, mechanical characteristics, and interactions.

Several key questions arise from our hypothesis:

- **Actin reorganization**: What drives and determines actin organization and reorganization? How does the reorganization of actin influence structure formation—is it leading or following the cell-external structures? Does actin constrain the secretion of cuticle precursor in a way that promotes the development of specific patterns?
- **Role of the epicuticular envelope**: What is the role of the epicuticular envelope in controlling cuticle precursor conformation? How does its growth and mechanical behavior change over time, and how do these changes affect scale morphogenesis? What maintains the close association between the epicuticular envelope and the cell membrane between ridges (defining the boundary of the soft compartment)? What is the epicuticular envelope made of? Does its composition differ, and explain variation in scale morphology?
- **Cuticle precursor variations**: How do differences in the chemical composition of the cuticle precursor affect its mechanical properties and hydration levels? How and where is the cuticle precursor delivered to the extracellular secretion compartments? How do the chemical composition and spatio-temporal variations in cuticle deposition influence the resulting form of ridges and other structures?
- **Process control and synchronization**: How are the physical properties and configurations of the actin cytoskeleton, soft compartments, and cuticle precursor encoded within the butterfly genome? How does the cell ensure synchronization of the intra- and extra-cellular processes that define the scale structures?

Below, we briefly discuss the most promising established as well as recently developed approaches and techniques that will aid in addressing these questions by making visible, modifying, and modeling the arrangement of the cellular machinery and the secretion of cuticle to shed light on the underlying genetics, mechanics, and process principles. Figure 11 shows examples of the techniques.

5.1 | Molecular, Genetic, and Multiomics Approaches

Building on the identified transcription factors that control scale structure differences, further work to understand the downstream targets of these genes could be a promising route for

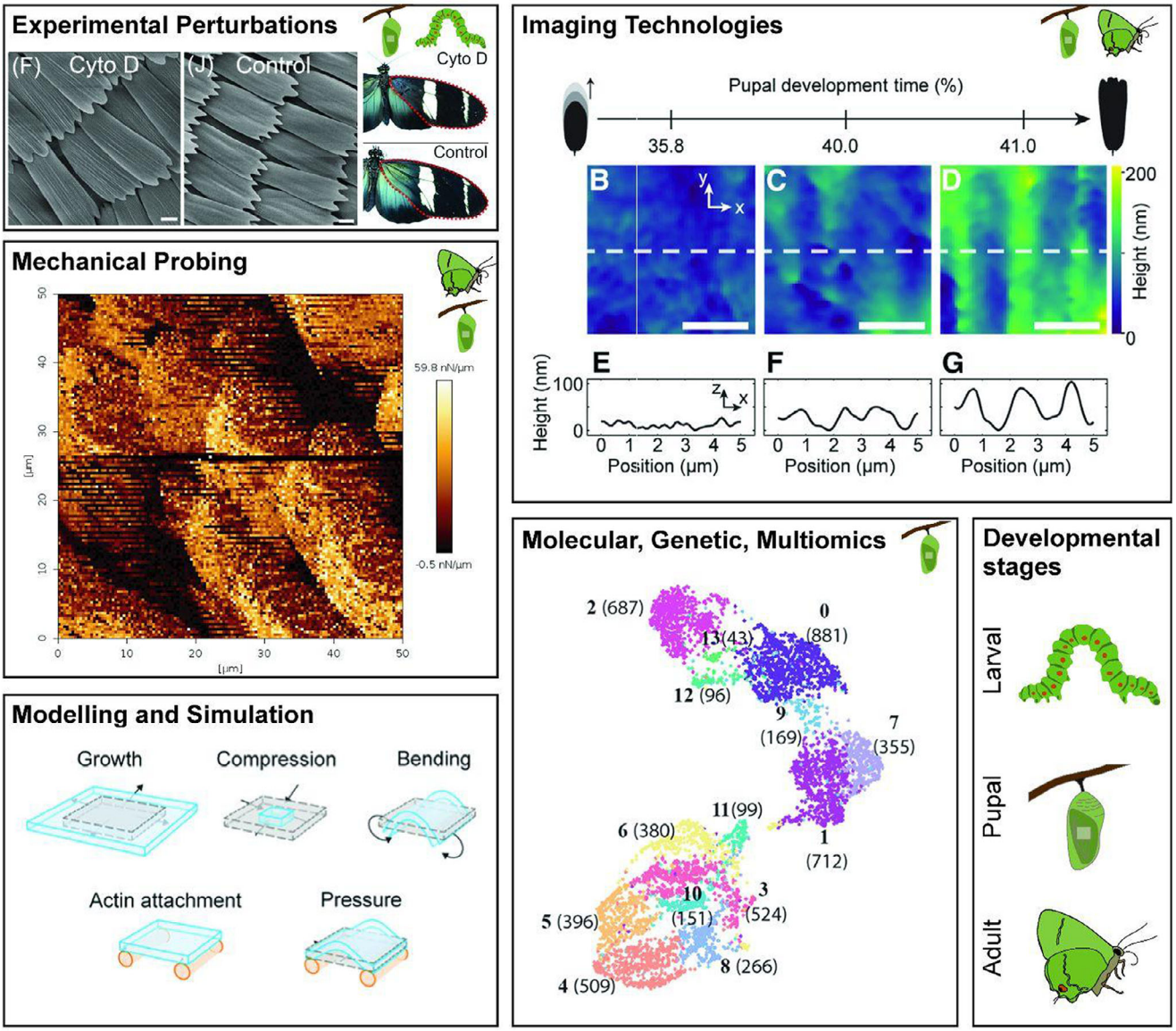

FIGURE 11 | Experimental and theoretical approaches for understanding butterfly wing scale growth. Methods can be applied to different species at various stages of butterfly development. For example, experimental perturbations at the larval or pupal stages can result in morphological changes seen in the adult butterfly. The example here is reproduced from ref. [30], where Cytochalasin D, targeting actin, was applied to pupal forewings affecting the development of wing scale ridges. Various imaging technologies exist, but few are compatible with in vivo imaging. The example presented shows the development of ridges using in vivo phase imaging. Molecular, genetic, and multiomics approaches provide promising avenues for characterizing the biochemical drivers of scale structure. Here we show an example from the ref. [92], where single-cell RNA sequencing was used to cluster ~5200 pupal forewing cells based on similarity in their gene expression profiles, to determine their molecular identity. We have also identified that mechanical probing may offer a route for testing how physical parameters affect the development of scale structures. Here, we show preliminary data from in vivo atomic force microscopy on a butterfly pupa that quantifies local stiffness across multiple wing scales. Finally, physical models and simulations provide a useful framework for understanding the physical parameters that may be at play during the development of a wing scale. Here we show an example of a ridge formation model as a consequence of cell membrane buckling due to growth, compression, bending, actin-membrane coupling, and the internal pressure of the cell [59]. All panels are reproduced with permission under CC-BY licenses.

identifying the genetic pathways directly responsible for controlling scale structure. Chromatin Immunoprecipitation Sequencing (ChIP-Seq) can be used to identify these downstream targets by using an antibody against the transcription factor of interest to pull down bound regions of DNA, which are then sequenced to identify the targets. Loh et al. did this for the Bab transcription factor, identifying genes likely involved in cytoskeletal dynamics and cuticle formation, amongst other things [87].

Advances in biological omics approaches, particularly sequencing and mass spectrometry [93], hold great promise for characterizing the genetic and biochemical differences between scale types and profiling these through development. Bulk RNA sequencing, comparing gene expression between tissues or individuals, has so far identified candidate genes associated with cytoskeletal organization [85, 86]. More recently, single-cell/nucleus sequencing has been used to profile the transcriptome of individual cells

from butterfly wing tissue [92] and to identify different scale types on the wing and transcriptional differences associated with these [87]. Applying this technique across a broader range of species will be useful for identifying common actors that differ in a consistent way between scales of specific morphologies. Going beyond sequencing dissociated single cells, spatial transcriptomics allows the transcriptome of individual cells to be characterized in situ [94]. As far as we are aware, this has not yet been attempted on butterfly wings (but see ref. [95]), and holds great promise for comparing different scale types on the wing, because the spatial information is retained. Without this spatial information, single-cell approaches require additional data or experiments to be able to link bioinformatically identified cell clusters to specific cell types on the wing.

While genomics and transcriptomics are excellent starting points for understanding how scale structure is controlled and the differences between cell types, these will not necessarily capture all of the differences in the downstream products determining scale structure. Mass spectrometry-based approaches to characterize differences in proteins (proteomics), lipids (lipidomics), and other metabolites (metabolomics) could prove fruitful for understanding the biochemical composition of the cuticle and how this differs between scales of different morphologies. Surprisingly little is known about the biochemical composition of scales, which has been assumed to be largely chitin, but this has not been directly tested, and some recent observations in particular call this into question [17]. Based on our hypothesis outlined above that the soft compartment between the epicuticular envelope and the plasma membrane is important for structure formation, understanding the composition of the epicuticular envelope is a key question, as is the composition of the cuticle precursor within this compartment, influencing the process by which this hardens and matures. Spatial multi-omics platforms using imaging mass spectrometry are also now a possibility [96, 97], and could allow spatial mapping across the wing of subtle variation in biochemical composition

5.2 | Imaging Technologies

Understanding butterfly scale growth and testing process hypotheses requires the direct visualization of the dynamic interplay between cytoskeletal organization, membrane mechanics, and cuticle maturation across development. High-resolution imaging approaches enable multi-scale interrogation of butterfly scale morphogenesis, from subcellular cytoskeletal dynamics to whole-scale architecture. Below, we discuss approaches that have already provided important insights into scale formation and some experimental methods that might be useful in the future.

Fluorescence microscopy techniques based on fluorescent labeling of two or more specific cell components simultaneously aid in identifying those components and revealing their spatio-temporal organization and interactions [98]. In some cases, bright-field optical microscopy can be used to identify cellular elements [99]. These specific cell components can include cell plasma- and endo-membranes, actin cytoskeletal components, trans-membrane proteins, and cuticle precursor ingredients. Fluorescent imaging methods can capture actin filament rearrangements, membrane undulations, and the initial deposition of cuticular precursors in developing scales, when suitable live imaging-compatible stains are chosen [98]. A major advantage of state-of-the-art fluorescence imaging approaches is high temporal resolution and minimal invasiveness, enabling in vivo tracking of cytoskeletal and membrane dynamics in time. Label-based approaches also confirm the identity of the components being imaged, while in label-free approaches, the molecular identity of structures is not known. However, limited penetration depth (5–20 μm), photobleaching, and optical aberrations in thick, pigmented pupal tissues can restrict the application of such approaches to earlier and/or surface-near developmental stages, with optical crowding prohibiting measurements at later time points. Fluorescence-based optical super-resolution microscopy—including stimulated emission depletion microscopy (STED), fluorescence lifetime imaging using STED principles (tauSTED), and structured illumination microscopy (SIM)—offers sub-100 nm resolution and live-imaging compatibility.

Quantitative phase imaging approaches – including speckle-correlation reflection phase microscopy – allow for label-free, minimal perturbation live imaging of developing tissues and have been shown to provide depth resolution down to 10 nm in some biological systems [100, 101]. This approach has been useful in visualizing scale formation continuously from the onset of pupation to eclosion. However, since the approach relies on refractive index variations and the ability to reconstruct the reflected wavefront, strong scattering in many tissue layers can reduce resolution. In butterflies, the live imaging resolution achieved in previous work amounts to around 1 μm [40].

Electron microscopy approaches—such as serial block-face scanning electron microscopy (SEM), transmission electron microscopy (TEM), and focused ion beam SEM (FIB-SEM)—provide near-isotropic 3D resolution (of about 5–20 nm) and exquisite morphological detail of cuticular ridges, cross-ribs, and honeycomb lattices, particularly in mature tissues. However, these methods require sample fixation and dehydration, precluding true in vivo imaging and capturing only static "frozen" morphologies. Nevertheless, sequential sampling of pupal stages can approximate developmental trajectories at ultrastructural resolution, as done in the seminal work by Ghiradella [15, 16]. In addition, approaches such as correlative light and electron microscopy (CLEM) combine the advantage that fluorescence imaging has in identifying molecular components of the scale with the resolution advantage that electron microscopy has for imaging the scale's architecture; such approaches may complement the fluorescence-based optical super-resolution microscopy techniques mentioned above [102, 103].

X-ray tomography (including nano-CT and ptychographic computed tomography) offers non-destructive 3D visualization of intact scales with sub-100 nm to few-hundred-nm voxel size and millimeter-scale field of view [18, 46, 104–106]. These studies demonstrated that synchrotron-based nano-CT can reconstruct hierarchical gyroid and multilayer architectures in dead and dried cuticle, providing essential "end-state" structural baselines. However, these techniques currently cannot capture in vivo dynamics: high photon flux, limited contrast in hydrated tissues, and radiation damage render live or partially hydrated

imaging impractical and tricky, especially as achieving true nanoscale resolution requires the addition of a cryo stage [104]. Novel approaches with low-dose, phase-contrast, or cryogenic tomography approaches may help bridge this gap by imaging semi-hydrated or freshly fixed pupal tissues ex vivo.

5.3 | Mechanical Probing

Mechanical probing offers a route to test how physical parameters contribute to butterfly wing scale morphogenesis. As argued above, during scale development, gradients in elastic modulus, surface tension, and viscoelastic relaxation times are likely to influence pattern formation and structural differentiation. Scanning probe techniques such as atomic force microscopy (AFM) can access several of these parameters directly—quantifying local stiffness (Young's modulus), adhesion forces, and surface topography at nanometer resolution [107, 108]. Applying AFM to developing or freshly eclosed scales could thus help reveal how mechanical heterogeneity or adjustments in mechanical stiffness patterns emerge in parallel with cellular or cuticular organization, providing data to test predictions from finite element models. However, ensuring stability and repeatability of the environmental conditions is particularly tricky with in vivo measurements using AFM as the probe has to be brought in contact with the wet sample.

Non-contact methods such as Brillouin microscopy can probe the longitudinal elastic modulus and viscoelastic response in vivo [109, 110], potentially offering a dynamic view of mechanical evolution in intact pupal tissues. Since Brillouin scattering is compatible with microscopic imaging [111, 112], it allows the correlation of mechanical measurements with molecular or cytoskeletal markers, helping to evaluate whether hypothesized stress or stiffness distributions occur during growth.

Additional approaches—such as micro-indentation for bulk stiffness assessment, optical tweezers for measuring local force responses [113, 114], or traction force microscopy for mapping the transmission of mechanical stress in the cell [115, 116] – could complement these observations, though their application to insect epithelia will likely be very challenging. Collectively, these tools may enable the cautious yet quantitative testing of how mechanical properties evolve during scale formation and how they contribute to the emergence of complex structural motifs.

5.4 | Modeling and Simulation

Computational modeling of mechanical instabilities provides a framework for understanding how variations in physical parameters shape butterfly wing scale morphology. By representing membranes as elastic shells, models at the continuum scale can capture how compressive stresses or differential growth lead to pattern formations [117, 118]. In addition, finite element (FE) simulation and molecular dynamic (MD) simulation offer complementary insights ranging from membrane-level deformation [119] to molecular-level lipid reorganization [120]. By systematically varying parameters such as bending rigidity, growth anisotropy, and surface tension, these models can predict morphological transitions and generate phase diagrams that link material properties to emergent structural forms.

For modeling at the continuum length scale, growth is commonly introduced kinematically through a multiplicative decomposition of the deformation gradient into growth and elastic components [121]. The spatial distribution and time evolution of the growth tensor may be prescribed phenomenologically, inferred from experiment, and can be further coupled to the evolving strain and/or stress fields in the material. The constitutive relation of the material is typically modeled as a hyperelastic material, such as a Neo-Hookean material. During growth, compressive stresses arising from differential growth can lead to mechanical instabilities [122, 123]. Many thin biological structures, such as plant leaves, flower petals, and insect wings, are described using Föppl–von Kármán plate theory within a morphoelastic framework [124]. With experimental data and images, such modeling and simulation can be used to reveal how mechanical forces and structural instabilities act as fundamental mechanisms explaining diverse structure formation in butterfly wing scales [59].

5.5 | Experimental Perturbations

Ultimately, to definitively prove any hypothesis about structure formation, manipulative experiments are needed. Genetic manipulations, such as gene knockouts or overexpression, are useful tools to test the role and function of specific genes and proteins on structure formation. CRISPR-Cas9 is now the primary method used for genetic manipulation in butterflies [125, 126] with the simplest and most common manipulation being gene knockouts. In most cases, mosaic knockouts are created, where a random subset of cells in an individual are affected. This has the benefit of allowing direct comparisons of wild-type and knockout phenotypes in the same individual, and possibly avoiding the lethal effects of some gene knockouts if not all cells are affected. However, a downside is that it can be difficult to verify the knockout in patches exhibiting a particular phenotype, particularly if those patches are small, as it requires sequencing DNA from those precise patches. The alternative, widely used in other organisms, is to create stable knockout lines [127], and to avoid lethal effects by using gene editing to alter expression levels or location through targeting regulatory regions of the gene of interest, rather than its coding sequence [128]. Another option for avoiding potentially lethal effects of knockouts is to target these to specific tissues and developmental stages, for example using RNAi [87]. Genetic manipulations targeting genes involved in actin organization, cuticle composition, secretion and hardening, and interactions between these, could allow for tests of the structure formation hypotheses we outline above.

Other forms of manipulative experiments can involve chemical treatments that target specific aspects of cell function or growth. Early work demonstrated that chemical perturbations can induce modifications in butterfly wing patterns and development [129]. Cytochalasin D is a chemical which inhibits actin polymerization and causes actin bundle disruption. Injections of this into developing wings at different developmental stages have demonstrated the importance of actin for scale growth and for correct ridge formation [30, 43]. Many pharmacological tools are available that

allow disruption or promotion of different components of the cytoskeleton [130] and could be used to test further hypotheses about the role of the specific components of the cytoskeleton at specific developmental time-points during structure formation, particularly when combined with live-cell imagining. A range of chemicals is also available that target specific aspects of the cuticle or its formation, developed for use as insecticides [131]. Previous studies have already used such approaches to probe butterfly wing development and color pattern formation [132]. Targeted use of these at low doses could be used to test the importance of different aspects of the cuticle and growth for structure formation. At a small and more precise scale, advanced optical techniques such as optical tweezers and laser ablation of individual actin bundles could be used to probe and manipulate individual sub-cellular components in live cells, for example, to release or apply tension [133] and determine how this influences forming extracellular structures.

Simpler manipulative experiments could involve changing the temperature or drying conditions during scale growth, an approach that has been previously used to modify butterfly wing color patterns and development [134]. Reaction speeds are expected to scale with temperature, so if structure formation processes are determined purely by physical processes, then temperature changes should cause predictable effects on the resulting structures. Alternatively, if the cell exerts a greater level of control over the formation process it may be able to buffer against temperature changes to some extent by up or downregulating expression of key components. Experiments to date suggest that scale structure formation processes are sensitive to temperature fluctuations [135]. An interesting future avenue could be to test whether models and simulations of structure formation with different reaction speeds can accurately predict observed differences in scale structures under different temperatures.

6 | Discussion and Conclusion

Our hypotheses emphasize the central role of mechanical instabilities in butterfly wing scale morphogenesis. We present a general overview of the progression of scale formation by integrating relevant biological structures, such as the actin cytoskeleton and plasma membrane, with biochemical processes like cuticle precursor secretion and sclerotization, and biomechanical phenomena such as buckling and deformation of soft secretion compartments. This integrative framework bridges molecular biology with biomechanics, offering a cohesive explanation for the extraordinary diversity of scale morphologies (as shown in Figure 2). This approach highlights the interplay between genetic factors, mechanical forces, and the properties of materials that ultimately shape the biological structures. In particular, this framework highlights how genetic control might define boundary conditions that harness mechanical instabilities due to competing local forces and time-dependent material properties for specific processes and structural outcomes [72, 75].

It is apparent that the actin cytoskeleton in forming butterfly wing scales is highly dynamic and reorganizes itself during the scale formation stages [30, 41]. Temporal changes in the actin configuration, coupled with the spatial and temporal heterogeneity of sclerotization, contribute to the intricate patterns and scale structures formed by mechanical instabilities. This interplay provides a plausible mechanism for generating ridges, crossribs, and other architectures with different levels of structural complexity.

The scale-forming cell across lepidopteran species solves an exciting problem tightly associated with cellular secretion: how to control the nano- and microscale morphology of matter that is secreted into an extracellular space. Cells can leverage distinct biochemical and mechanical processes to structure their intracellular environment [78]. However, a substantially smaller set of controls is available for controlling the extracellular physical and chemical processes that ultimately determine the final solidified morphology of the secreted materials. As discussed in Section 4, these handles are likely restricted to the spatio-temporal composition of the involved precursors, the rate of precursor secretion, and the growth rate of templating compartments. While our view is supported by existing observations and theoretical principles, several limitations must be acknowledged: a direct, real-time visualization of the dynamic processes during scale development presented in Section 4 remains challenging due to technical constraints in imaging living tissues at the necessary spatial and temporal resolutions. Future research should focus on developing real-time, high-resolution imaging techniques and experimental methods to observe and perturb these processes in vivo.

Understanding the fundamental phenomena involved in the cell's control of extracellular structure formation processes is relevant beyond the specific context of butterfly scales, as it holds important lessons for understanding extracellular secretion more broadly and for designing sustainable materials. Many biological systems rely on secretion into defined spaces followed by material maturation to achieve a mechanically-driven functional pattern, including pollen cell walls, bone, and insect egg chorions [136, 137]. Organisms such as diatoms, which form intricate silica shells, and mollusks [138, 139], which build complex shells through biomineralization processes, may also utilize similar mechanisms. Exploring these parallels may reveal conserved strategies by which cells shape extracellular materials across diverse taxa.

Beyond biology, insights from studying natural systems have the potential to inspire biomimetic applications in materials science and engineering [140]. Natural systems demonstrate how complex micro- and nanoscale architectures are fabricated without top–down patterning in ambient conditions by instead exploiting mechanical instabilities, confinement, and controlled temporal variation of material characteristics. Translating these principles into synthetic systems could inspire new fabrication strategies for functional and sustainable materials.

In this perspective, we propose that mechanical instabilities arising from the interplay between a soft compartment constrained by the actin cytoskeleton and the sclerotizing cuticle precursor are fundamental to the diversity of observed butterfly wing scale structures. We emphasize the critical roles of mechanical forces and material properties, alongside genetic and biochemical regulation in morphogenesis. The hypotheses in this perspective have significant implications for developmental biology. They provide a framework that bridges genetically-controlled

molecular mechanisms with chemical and physical processes. By elucidating how complex structures can arise from the interplay of cellular components and mechanical instabilities, we open new avenues for interdisciplinary research. We expect that insights into the principles and processes allowing organisms to control the structure and composition of their secreted functional materials have potential for broader impact in biomaterials design and engineering. Specifically, understanding the biomechanical phenomena harnessed in biological secretion could inform the design of advanced materials and fabrication methods. Future investigations aimed at validating and expanding the hypotheses expressed in this perspective – defined around single-cell secretion of butterfly scales – will not only deepen our understanding of butterfly scale morphogenesis but may also uncover universal principles driving secretion of functional materials in biology that may inform technological applications and sustainable materials design.

Glossary (Biology)

Epicuticular envelope – The superficial strata of the scale cell, often waxy and hydrophobic, secreted early during cuticle formation as a layer separating the cell from the haemolymph. It can contain up three layers visible in electron microscopy and provide a scaffold for subsequent deposition of cuticle. Hypothesized to function in preventing water loss. Likely anchored locally to the plasma membrane and/or to the cellular actin network via transmembrane proteins.

Cuticle composition – The insect cuticle is composed of chitin embedded in a matrix of cuticular proteins (including resilin, structural glycoproteins, and cross-linking proteins) and may also contain lipids and sometimes sclerotizing agents, such as catecholamines. Pigments (e.g., melanin, pterins, ommochromes, flavonoids) may be incorporated into the cuticle matrix. The relative abundance and molecular organization of these components determine mechanical stiffness, flexibility, and optical function. Differences likely occur within species.

Butterfly scale components – The nanostructures that define butterfly wing scale architecture: **Ridges** – Longitudinal, regularly spaced structural elements, often found on the upper surface of scales. Ridges providing structural support, determine the wetting characteristics of scales, and in many species have optical function, for instance acting as spectrally-selective reflectors. **Cross-ribs** – Fine transverse connections between ridges, supporting membrane-like regions. **Lamellae** – Overlapping chitin sheets stacked beneath ridges; often act as multilayer reflectors producing structural coloration. **Trabeculae** – Vertical pillars connecting the upper and lower lamina, maintaining spacing and lattice stability. **Lower lamina** – A basal thin film that can act as an optical reflector. Often unstructured and directed towards the wing.

Sclerotization (tanning) – The biochemical process that cross-links cuticular proteins and catechols, hardening and darkening the cuticle. Sclerotization modifies scale rigidity and influences light absorption.

Actin network – A dynamic cytoskeletal framework composed of filamentous actin (F-actin). In butterfly scale cells, actin filaments form elongated longitudinal bundles that prefigure ridge spacing and orientation, acting as templates for cuticle deposition. Remodeling of actin is also implicated in shaping lamellae and other fine nanostructures later in the scale formation process.

Microtubule network – Cytoskeletal polymers of α- and β-tubulin dimers that radiate from organizing organelles within scale cells. Microtubules provide intracellular transport pathways for vesicles and cuticular material, help establish cell polarity, and coordinate with actin filaments during ridge and trabeculae formation.

Transcription factor - A protein that controls the process of transcribing a gene from DNA to RNA by binding to specific regions of the DNA sequence, often, but not always, close to the target gene.

Glossary (Physics)

Buckling – A mechanical instability in which thin or slender structures under compressive stress deviate from their original axis or plane. In butterfly wing scales, buckling of cuticular sheets or lamellae can produce periodic folds and corrugations that contribute to ordered nanostructures.

Finite element modelling (FEM) – A computational method that divides complex structures into discrete elements to simulate mechanical behavior under stress and strain. FEM is used in biological physics to model how layers deform, buckle, or wrinkle during formation or application of external stresses.

Mechanical instabilities – Spontaneous deformations or pattern formations that arise when internal or external forces exceed structural stability thresholds.

Elastic modulus – A measure of a material's stiffness, defined as the ratio of stress to strain within the elastic (reversible) deformation range.

Self-organization – Spontaneous emergence of ordered structures from local interactions and physical constraints, without a centralized template or control.

Wrinkling – A periodic surface deformation resulting from compressive stresses, typically producing sinusoidal patterns. Wrinkling of cuticular membranes in scale cells may precede or accompany buckling in the generation of nanoscale textures.

Folding – A higher-amplitude mechanical instability compared to wrinkling, often producing sharp bends or creases in a material.

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Conflicts of Interest

The authors declare no conflicts of interest.

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