Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Evolution of Developmental Control Mechanisms

Dynamics of F-actin prefigure the structure of butterfly wing scales

April Dinwiddie^{a,*}, Ryan Null^b, Maria Pizzano^b, Lisa Chuong^b, Alexis Leigh Krup^b, Hwei Ee Tan^b, Nipam H. Patel^{b,*}

^a Department of Ecology and Evolutionary Biology, Yale University, New Haven, CT 06511, USA ^b Department of Molecular and Cell Biology & Department of Integrative Biology, University of California Berkeley, Berkeley, CA 94720-3200, USA

ARTICLE INFO

Article history: Received 22 March 2014 Received in revised form 2 June 2014 Accepted 4 June 2014 Available online 12 June 2014

Keywords: Lepidoptera Macrochaetes F-actin Butterfly scale ultrastructure

ABSTRACT

The wings of butterflies and moths consist of dorsal and ventral epidermal surfaces that give rise to overlapping layers of scales and hairs (Lepidoptera, "scale wing"). Wing scales (average length $\sim 200~\mu m$) are homologous to insect bristles (macrochaetes), and their colors create the patterns that characterize lepidopteran wings. The topology and surface sculpture of wing scales vary widely, and this architectural complexity arises from variations in the developmental program of the individual scale cells of the wing epithelium. One of the more striking features of lepidopteran wing scales are the longitudinal ridges that run the length of the mature (dead) cell, gathering the cuticularized scale cell surface into pleats on the sides of each scale. While also present around the periphery of other insect bristles and hairs, longitudinal ridges in lepidopteran wing scales gain new significance for their creation of iridescent color through microribs and lamellae. Here we show the dynamics of the highly organized F-actin filaments during scale cell development, and present experimental manipulations of actin polymerization that reveal the essential role of this cytoskeletal component in wing scale elongation and the positioning of longitudinal ribs.

© 2014 Elsevier Inc. All rights reserved.

Introduction

Butterfly wings have played a pivotal role in the discovery and study of many major evolutionary, behavioral, and ecological processes. In recent years, significant advances have been made in understanding the developmental underpinnings that generate lepidopteran wing patterns. Most of these studies have focused on the control of early pattern elements on the wing (e.g. eyespots and veins) (Nijhout, 1991; Carroll et al., 1994; Beldade et al., 2002; Stoehr et al., 2013), genes that control variation in color patterns between species (Joron et al., 2011; Reed et al., 2011; Martin et al., 2012), and mathematical or optics-based models of how some structural colors are produced (Vukusic et al., 2000; Stavenga et al., 2004; Kolle et al., 2010; Saranathan et al., 2010). What is missing in our current picture of lepidopteran wing patterning is an understanding of the developmental process that gives an individual wing scale cell, which is the primary unit for butterfly wing color, its particular shape and creates the intricate cuticular structures that pattern its surface and modulates or creates its individual color (Fig. 1).

Bristles and scales (also known as macrochaetes) arise from similar developmental programs, and are distinct from fine insect

* Corresponding authors. *E-mail addresses:* april.dinwiddie@yale.edu (A. Dinwiddie), nipam@uclink.berkeley.edu (N.H. Patel).

http://dx.doi.org/10.1016/j.ydbio.2014.06.005 0012-1606/© 2014 Elsevier Inc. All rights reserved. hairs (microchaetes); the former have sockets at their base, while the later lack sockets. Bristles and scales perform similar functions, but their shapes are distinct: bristles are conical, with cuticular ridges winding around their periphery, while scales are air filled sacs between two sheets of chitin, known as laminae. Bristles are found in all arthropod groups, while scales are predominately found within the hexapods, though they are also produced in some arachnids (Ghiradella, 2010). In the Lepidoptera, the lower lamina of a scale cell is generally (though not always) smooth, while the upper lamina is organized into longitudinal ridges that run the length of the scale. In some species, finely spaced microribs at the sides of longitudinal ridges, are capable of specifically scattering light to create structural color (Vukusic et al., 1999).

Macrochaetes have been most extensively studied in *Drosophila*, where they have been shown to arise from a single epidermal cell type. The production of one macrochaete involves five specialized cells derived from the sensory organ precursor cell (SOP cell) (Colombani et al., 2005). During development, the SOP cell undergoes two asymmetric divisions, and the daughter cells differentiate into the components of the bristle or scale: shaft, socket, internal neuron, glial cell, and sheath (Roegiers et al., 2001; Held, 2002). Macrochaetes share similar genetic programs during development. The homolog of the transcription factor *achaetescute*, which promotes *Drosophila* bristle precursor formation, is also expressed in scale and socket progenitors of Lepidoptera









Fig. 1. Wing scales of the painted lady butterfly, *Vanessa cardui*. (A) *V. cardui* is a common lepidopteran species found throughout the world. Scale bar=1 cm. (B) The wing is covered in overlapping layers of scale cells as seen in this reflected light image in the region of one of the ventral hindwing eyespots. The scales themselves are composed of chitin, but in the adult are no longer alive, rather just the remains of once living cells. Scale bar=2 mm. (C) Higher magnification image of wing scales. The scales are arranged like overlapping tiles. Note that each scale is just a single color. The wing scales all point toward the proximal end of the wing (away from the body), which in this image is oriented down. Scale bar=150 μ m. (D) SEM image showing finger-like projection (green arrow) at the end of scale cells and the socket (blue arrow) where scales are attached to the wing epithelium. Scale bar=65 μ m. (E) SEM of base of scale cell where the scale shaft (red arrow) inserts into the socket cell (blue arrow) Scale bar=20 μ m. (F) High magnification SEM showing that scale cell surfaces are ornately patterned with longitudinal ridges (yellow arrow) and cross-ribs between the ridges. Scale bar=2 μ m. (G) 6-day pupal wing scale stained with fluorescent Wheat Germ Agglutinin (WGA), a lectin that binds to chitin. Green arrow indicates finger like projections, and red arrow indicates the shaft of the scale (scale has been pulled from the socket). By this stage of development, pupal scales already have the overall size and morphology characteristic of adult scales. Scale bar=20 μ m.

(Galant et al., 1998). What we refer to as either a scale or a bristle is a cellular extension, derived from the shaft cell (Figs. 2 and 3G). During larval development in Lepidoptera, wing imaginal discs enlarge, veins form, and basic gross pre-patterns (eyespot prepatterns, etc.) are established through the expression of transcription factors and signaling molecules. It is not until the pupal stage, however, that the morphological development of wing scales begins.

In lepidopteran development (at the pupal stage), each scale cell body, located within the wing epithelium, produces a cytoplasm-filled extension that is surrounded by an active cell membrane that extends out of the wing epithelium (Fig. 2). The extensions begin as cylinders that become flattened during growth. The scale cell extension grows enormously and quickly during development, ultimately producing the surface extension that we recognize as an adult wing scale. The size of wing scales is shown to correlate with the size of the wing-blade (Simonsen and Kristensen, 2003), and the degree of scale cell polyploidy (as scale cells undergo several endomitotic divisions) (Greenstein, 1972; Cho and Nijhout, 2013). In the late stages of pupation the scale cell dies, leaving a non-living skeleton of chitin filled with air and pigment.

The morphological differences between scales and bristles likely result from components or modifiers of the cytoskeleton and cell membrane. Actin bundles outlining the cell periphery are thought to set the shape of a bristle. These bundles have been extensively studied in the formation of *Drosophila melanogaster* thoracic bristles, and the actin structures are essential for elongation and curvature (Tilney et al., 2000; Guild et al., 2005; Tilney and DeRosier, 2005). Turner and Adler (1998) found that treatment of developing *Drosophila* bristles with cytochalasin-D, an inhibitor of actin polymerization, affects bristle formation, producing bristles that are stunted or bent (and occasionally branched). Tilney et al. (2000) also inhibited growth of *Drosophila* bristles with cytochalasin-D, and determined that actin filaments are essential for bristle elongation. A reinvestigation of the role of microtubules in *Drosophila* bristle elongation indicates that two populations of microtubules help to guide bristle development: dynamic microtubules (with mixed polarity) add bulk to the bristle cytoplasm, while stable microtubules (with minus ends distal to the cell body) act to polarize the axis of bristle elongation (Bitan et al., 2010, 2012).

Thus an understanding of *Drosophila* bristles offers a starting point from which we can further study the developmental events producing a wing scale. One of the first studies of insect macrochaete development determined the orientation of different molecules within developing fly bristles (Lees and Picken, 1945), and then Picken (1949) extended this work into lepidopteran wing scales and predicted the periodic distribution of what we have found to be actin bundles. More recently, one additional ultrastructural element has been suggested as a part of the story: where actin filaments (or "microfibrils", as termed in Greenstein, 1972) are attached to the cell membrane (termed areas of close contact "cc" (Locke, 1967; Greenstein, 1972), cuticle cannot be secreted, and this could contribute to the production of longitudinal ridges in lepidopteran wing scales. Ghiradella argues that



Fig. 2. Initial formation of scale and socket cells and extension of the scale (V.cardui). (A) CellMask plasma membrane stain (magenta; see section "Materials and methods") at 7% of pupal development, which is 12 h after pupa formation (APF), reveals the SOP cells (arrow), the precursor cells to the scale and socket cells. Scale bar=30 µm. (B) 3D view reveals that the SOP cells bulge upward from the surface. (C) At about 14% of pupal development (24 h APF) the SOPs have divided and phalloidin staining reveals the alignment of scale cells (arrow) arranged in rows in the wing epithelium. Scale bar=30 µm. (D-D") 21% of pupal development (36 h APF) stained for beta-catenin (white), DAPI, and Ubx. Scale bar=15 µm. (D) At 36 h APF, beta-catenin (white) highlights the apical surface of the wing epithelial cells and reveals the outline of the C-shaped socket cell (arrow) and narrow, circular outline (arrowhead) of the shaft of the scale, which is wrapped by the socket cell. Wing is oriented with the distal end of the wing pointed down. (D') Below the wing surface are the nuclei of the socket (green nuclei) and scale cells (magenta nuclei), which are organized into rows with intervening bands of other wing cell nuclei (blue, stained with anti-Ubx, see text). The scale nuclei sit well below the apical surface while the socket nuclei sit close to the apical wing surface. (D") Overlay of beta-catenin pattern (white) with the most superficial nuclei reveals that the socket nuclei (green nuclei) are positioned just distal to the shaft outline. (E-E") 21% of pupal development (36 h APF) stained for beta-catenin (white), DAPI (blue), and phalloidin (yellow). Scale bar = 5 µm. (E) Single z section of beta-catenin staining (white) catches outline of socket (arrow) and scale shaft (arrowhead) lying just above the rest of the wing epithelium at 21% of pupal development (36 h APF). Below are the large nuclei of the scale and socket (blue). (E') Magenta arrow indicates scale nucleus and green arrow points to socket nucleus. Phalloidin staining (yellow) reveals the very early stage of extension of the scale out of the wing epithelium. (E") Overlay of beta-catenin (white) pattern and phalloidin staining (yellow) reveals that scale starts out extending distally as it comes up out of the wing epithelium. (F) At 36 h APF, phalloidin staining (yellow) reveals the orderly arrangement of scales, and the alternation between larger cover scales (arrow) and smaller ground scales (arrowhead). Scale bar=15 µm. (G) At 23% of pupal development (38 h APF), scales continue to extend from the wing epithelium (phalloidin staining in yellow). Here the wing was purposely folded over so that scales would project away from the surface. Scale bar = 15 µm. (H) Super-resolution (Stimulated Emission Depletion, STED) imaging of phalloidin staining at 23% of pupal development (38 h APF) reveals that F-actin is beginning to organize into bundles oriented along the proximal-distal axis of the scale. Scale bar=5 µm. (I) Side view (yz plane) at 24% of pupal development (40 h APF) with distal end of wing oriented down and apical surface of epithelium oriented to the left. The large, polyploid nucleus of the scale (magenta arrow) is located deeper into the epithelium than the socket nucleus (green arrow). The green arrowhead points to the socket and the magenta arrowhead points to the scale, which are both highlighted by phalloidin staining (yellow). Scale bar $= 5 \,\mu m$.

microfilaments (term for actin filaments) and their associated "close contact" regions contribute to determining the location of the ridges that will form between the bundles, and the windows that will form over the bundles (Ghiradella, 1998).

Here we apply confocal microscopy to generate a detailed description of the dynamic organization of F-actin during butterfly

scale development. Our analysis provides a greatly improved picture of the three-dimensional organization of the scale cytoskeleton during development and reveals both similarities and differences to what has been described for *Drosophila* bristle development (see Tilney et al., 2000). From these observations we can suggest the roles that the actin cytoskeleton might play in



Fig. 3. Formation of highly organized F-actin bundles within scales (Junonia coenia). (A-A") Developing wing scales at 28% of pupal development (72 h APF, developmental time is slightly longer for J. coenia than in V. cardui; this would be comparable to 48 h APF for V. cardui). WGA (magenta) stains cell membranes and phalloidin (yellow) stains F-actin. Scale cells have grown rapidly and now contain a multitude of fine actin bundles that are highly organized along the proximal-distal axis of the scale. For the actin bundles that do extend close to the distal most end of the scale, the phalloidin staining intensity weakens toward the distal end of the scales, and WGA staining is more intense toward the distal tip. Scale bar=15 µm (see also Supplementary Movie 1). (B-B") xz cross-section through scales shown in A-A" reveals that actin bundles are organized just under the cell membrane, and that phalloidin staining is more intense on the bundles that run along the lower surface of the scale. This intensity difference is most notable in the more proximal parts of the scale. Scale bar=5 µm (see also Supplementary Movie 2). (C-C*) 32% of pupal development (80 h APF) J. coenia wing scale cells (equivalent to 54 h APF in V. cardui) stained for WGA (magenta; cell membrane) and phalloidin (yellow, F-actin). Scales have continued to elongate, and the actin bundles have become further organized into thicker bundles. Scale bar=15 µm. (See also Supplementary Movie 3; to see how these scales relate to the position of underlying sockets, cell bodies, and nuclei, see Supplementary Movie 4.) (D-D") Cross sections (3D projections) through wing scales of C-C". Peripheral actin bundles have decreased in number, but increased in diameter, so that now individual bundles appear as distinct "rods" within the scale. Bundles on the underside of the scale are more intensely stained by phalloidin (arrowhead in D'). In addition, approximately 6-12 "rods" are located further internal to the cell membrane, but only on the lower surface of the scale (arrow in D'). These internal bundles only extend about 2/3rds of the way along the proximal-distal axis and do not reach the distal end of the scale. Scale bar=5 μ m (see also Supplementary Movie 5). (E) Phalloidin staining near the base of the scale reveals the socket structure (arrowhead) and the F-actin bundles of the scale that pass through the socket (arrow) at 32% of pupal development (80 h APF for J. coenia). Scale bar = 15 µm. (F) Optical cross section again shows sockets (arrowhead), and that F-actin bundles that pass through the socket extend underneath the epithelial surface and into the cell body of the scale cell (arrow). Scale bar=15 µm. (G) Schematic diagram of scale socket structure (32%/80 h APF for J. coenia and 54 h APF for V. cardui). Outline of scale and sockets shown in purple, nuclei in blue (socket nucleus=green arrow; scale nucleus=magenta arrow), and peripheral actin bundles in red and internal actin bundles in green.

scale development. We use cytochalasin-D treatment to experimentally manipulate the actin cytoskeleton and test our hypotheses. We also present preliminary data on differences in scale development between different wing regions in a single species, and between butterfly species. Our results, along with the methods we have developed, provide the groundwork for further studies of scale shape determination and the generation of scale nanostructures that lead to structural coloration in Lepidoptera.

Materials and methods

Butterfly stocks and developmental staging

An original stock of Painted lady (Vanessa cardui - Fig. 1) larvae was purchased from Carolina Biological Supply. Additional larvae were regularly obtained from Shady Oak Butterfly Farm in Brooker, Florida. Larvae were maintained on Stonefly Heliothis Diet Formula (Ward's Scientific) at room temperature, with a natural light cycle. Adults were kept in egg laying cages and were fed 5% sugar water. Eggs were collected on both mallow and hollyhock plants. Buckeye butterfly (Junonia coenia) larvae were obtained from Shady Oak Butterfly Farm and were raised on ribgrass plantain (also purchased from Shady Oak Butterfly Farm). Cabbage white butterfly (Pieris rapae) larvae were purchased, along with food, from Carolina Biological Supply. Zebra longwing (Heliconius charithonia) pre-pupae were purchased from Magic Wings Butterfly House in Deerfield, Massachusetts. Morpho peleides and Cattleheart (Parides arcas/iphadamas) pupae were purchased from Costa Rican suppliers, and were dissected upon arrival at the McGuire center for Lepidoptera and Biodiversity in Gainesville, Florida. An additional shipment of Cattleheart pupae was purchased and shipped to Yale University from Denver Pupal Supply. Greater wax moth (Galleria mellonella) larvae were purchased from Carolina Biological Supply, and Gulf fritillary (Agrulis vanillae) larvae were obtained from butterfly hobbyists in California.

All animals (unless shipped as pupae) were staged from the point of pupal case formation. Prepupae were collected and placed in front of a time-lapse camera so that exact pupation times could be recorded. Newly formed pupae were placed in an incubator at 22 °C. At the appropriate time of development, the pupa was removed and its wings dissected; age was recorded as hours after pupal case formation (h APF).

Dissection of pupae and staining of pupal wings

Time series with CellMask Plasma Membrane stain

Each pupal wing was dissected into 1X Grace's Insect Culture Medium (GIBCO 11300-043). Immediately after dissection, the wings of each pupa were fixed and stained as follows: The right forewing was treated with a cell membrane stain (CellMask Plasma Membrane Stain, Invitrogen C10046) diluted 1:1000 in $1 \times$ Grace's Insect Culture Medium for 10 min and was fixed in 4% PEM-PFA for 15 min. The left forewing was immediately fixed in 4% PEM-PFA for 15 min and was then incubated with phalloidin, Alexa 555 conjugated (Invitrogen A34055) at a dilution of 1:200 in PT overnight at 4 °C. The left hindwing was treated with an ER Stain (ER-Tracker, Invitrogen E34251) diluted 1:1000 in $1\,\times$ Grace's Insect Culture Medium for 30 min and was fixed in 4% PEM-PFA for 15 min. The right hindwing did not receive a stain - it was used as a negative control, and it was placed in $1\times\,$ Grace's Insect Culture Medium for 10 min and then was fixed in 4% PEM-PFA for 15 min. After staining, all disks were stored in 70% glycerol in $1 \times PBS$.

Time series with Wheat Germ Agglutinin and phalloidin

Wheat Germ Agglutinin (WGA), which binds to specific carbohydrates, has been previously used to examine cuticle development in other insects (Merzendorfer and Zimoch, 2003; Farnesi et al., 2012). We have found that WGA is also a useful probe for visualizing early scale bud formation and growth. As in *Drosophila*, WGA stains the nuclear envelope, but as development proceeds, it highlights the cell surface membrane, and then ultimately strongly stains the cuticle as it is secreted. Thus, WGA is useful to track and observe numerous stages of scale development.

Phalloidin is isolated from the fungus, *Amanita phalloides*. Staining with phalloidin (conjugated with a fluorescent molecule) is a standard method to visualize filamentous actin (F-actin) within cells; phalloidin binds to and stabilizes actin filaments. Double staining with phalloidin and WGA allows for optimal visualization of how the cytoskeletal components interact within the scale cell to help create structures.

Each pupal wing was dissected into and fixed in 4% PEM-PFA for 15 min. The left wings were then incubated in phalloidin, Alexa 555 conjugated (Invitrogen A34055) at a concentration of 1:200, and Wheat Germ Agglutinin, Alexa 647 conjugated (Invitrogen W32466) at a concentration of 1:300 overnight at 4 °C. Wings were stained for DAPI in 50% glycerol–PBS with 1 μ g/mL DAPI, and incubated overnight at 4 °C. Right wings were used for control purposes and were not stained, but were fixed and incubated in PT. This same procedure was used to stain the wings produced by the actin inhibitor experiments.

Antibodies

Rabbit anti-beta-catenin was obtained from Sigma Chemical Co. (St. Louis, MO) and used at a dilution of 1:1000. Ubx in the hindwings was detected with the anti-Ubx/abdA monoclonal F96.87 (Kelsh et al., 1994) used at a dilution of 1:30.

Injection set-up and experimental procedure

The cytochalasin-D (Cyto-D) was obtained from Sigma Chemical Co. (St. Louis, MO). Stock solutions were prepared in DMSO and stored as aliquots at -20 °C. For pupal injections, Cyto-D was diluted to the final concentration of 20 μM in 1 $\times\,$ Grace's Insect Medium.

Three *V. cardui* pupae were injected at each time point (12, 24, 30, 36, 42, 48, 54, 60, 66, 72, 78, 84, 90, and 96 h APF). Control pupae of these same ages were injected with a comparable concentration of DMSO in Grace's insect medium. Pupae were allowed to develop until 96 h APF and were then dissected into 4% PEM-PFA and stained (as indicated above). Each pupa was injected above the wing blade and into the bloodstream. Pupae were injected using a Hamilton Company syringe (CAT #7659-01) and custom ordered needles (28Guage, 30 mm, Point2). Each pupa was injected with 5 μ L of diluted drug (or control solution) on each side (10 μ L total).

Analysis of stains by confocal microscopy

Wing samples were placed on microscope slides and mounted in 70% glycerol: $1 \times PBS$. A coverslip (#1.5 thickness) was applied, and each preparation was sealed with nail polish. Slides were examined with Carl Zeiss model CLSM 700 and 710 (Yale University) confocal microscopes. For super-resolution microscopy we used Leica TCS SP8 Gated STED and Leica SR GSD 3D microscopes at the Leica facilities in Mannheim and Wetzlar, Germany respectively. Confocal images were examined and images and movies generated using Perkin Elmer Volocity 3D Image Analysis Software. Figures were assembled using Adobe Photoshop and movies processed and compressed with Quicktime 7 Pro.

Autofluorescence

Wing scales begin to autofluoresce when cuticle (composed of chitin) is laid down. We find that initially this autofluorescence is strongest with excitation by short wavelengths, but as development proceeds, the autofluorescence becomes stronger at longer wavelengths as well. We can use this autofluorescence to our advantage for some experiments, as we can visualize cuticle quite readily employing the standard settings used for DAPI fluorescence. However, we also note that settings used for green fluorescence (such as for Alexa488) quickly become compromised by cuticle autofluorescence. While linear unmixing of the signal readily eliminates this autofluorescence, we simply avoided these wavelengths. Thus in our experiments with older stages where cuticle is present, we used only Alexa555 and Alexa647 conjugates of phalloidin, Cell Mask, and WGA. By very late stages, even the 555 nm channel overlaps with autofluorescence, and comparison to negative controls is absolutely essential.

Scanning electron microscopy

Small squares of *V. cardui* wings were cut using a razor blade. These squares were mounted with carbon tape to aluminum stubs. In addition, wing scales were brushed off of *V. cardui* wings with a small paintbrush and mounted with carbon tape onto aluminum stubs. All stubs were coated with a layer of \sim 15 nm gold to increase sample conductivity. All SEM images were taken using a SU-70 UHR Schottky (Analytical) FE-SEM (Hitachi High 40 Technologies America, Inc.) at 10 kV accelerating voltage and 28 µA probe current.

Results and discussion

Our time series covers wing scale development of *V. cardui* from 12 h to 108 h after pupal case formation (APF, see section "Materials and methods" regarding timing of pupal formation). We have also examined wing scales at a few selected time points in a number of additional butterfly species: *J. coenia*, *P. rapae*, *P. arcas/iphadamas*, *M. peleides*, *H. charithonia*, and *A. vanillae* as well as the pyralid moth species, *G. mellonella*. As wing scale development was comparable in all of the species studied (see below), unless stated otherwise, we report our results from observations of the painted lady butterfly, *V. cardui* (Fig. 1). We most commonly used a combination of phalloidin to stain F-actin within the developing cells and Wheat Germ Agglutinin (WGA) to outline the cell membrane of scale cells.

The average timeline from pupation to eclosion (adult emergence) for V. cardui at 20 °C is 7 days, and we report our time series findings in both hours APF and as a percentage of developmental time (i.e. the percentage of 7 days completed) to facilitate comparisons to species that develop at different rates. In different wing regions the rate of scale elongation can differ; a general principle is that longer scales will bud earlier than shorter scales, and they will also elongate more quickly. For example, the scale cells along the wing edge, which tend to be longer and more bristle-like, exhibit a much faster rate of elongation than the smaller scale cells toward the middle of the wing. In addition, we observed a faster rate of macrochaete elongation in the hindwings, which generally contain more bristle-like scales. For consistency, the findings that we present in this paper were observed in scale cells (non-bristle-like) located between the M2 and M3 veins near the discal cell border of the forewing. This allowed us a more representative sample of the most common scale type on the wings (i.e. neither edge scales, nor androconial scales).

Initial scale and socket formation and scale elongation

During larval stages no discernible scale or socket cells, or their precursors, are visible, rather the epithelium is a sheet of rather uniform looking cells. At about 7% of pupal development (12 h APF for V. cardui), however, the SOP cells, which are the precursors to the scale and socket cells, do appear (Galant et al., 1998; Fig. 2A) and are organized in clear rows aligned parallel to the A-P axis of the wing. By 14% of pupal development (24 h APF for V. cardui), the scale and socket cells are visible (Fig. 2C, D-D'', E-E'', and I). The scale cell is highly polyploid, and the cell body lies internally within the wing (further in from the apical surface) (Fig. 2I). A second, polyploid cell, the socket cell, is associated with each scale cell. Socket cells are just distal to the scale cell and are more superficially located in the wing epithelium relative to the bulk of the scale cell body. The socket cell partially wraps around the part of the scale cell that is at the upper (apical) surface of the wing epithelium, giving its outline a "C" shape (Fig. 2D, E, and E"). While Ultrabithorax (Ubx) is uniformly expressed in hindwing cells at the larval stage, we find that it is no longer expressed by scale and socket cells during the pupal stage. Thus, Ubx staining in the hindwing can be used to identify all non-socket and non-scale cell nuclei. The scale nuclei are easily distinguished as they are the largest nuclei, and sit far deeper in the wing than any other cells. The socket nuclei can be identified as the more superficial nuclei that are Ubx-negative and have a characteristic oval shape (see Fig. 2D-D").

Similar to *Drosophila* bristles, the scale cells develop as small buds containing densely packed F-actin filaments. Elongation of the scale cells begins with many short parallel actin filaments (Fig. 2H), but these quickly form a smaller number of very thick bundles (Fig. 3) – precisely oriented and very regularly spaced. In many areas of the adult wing, there are alternating ground and cover scales, with the ground scales usually being shorter than the cover scales. We can see this distinction from early pupal stages as well (Fig. 2F). In some areas of the wing, particularly in the posterior dorsal surface of the hindwing, there are both typical scale cells as well as cells that are much more bristle-like, which form the "hairy" regions seen in this part of the adult wing.

Lees and Picken (1945) and Tilney et al. (2000) demonstrate that elongation of macrochaete bristles occurs by tip growth in *Drosophila*. Tilney et al. (1995) suggest that subunits of actin are rapidly added to the barbed ends of the actin filaments of *Drosophila* bristles, and that the cross-linking protein Formin is required early to bring filaments together. A second cross-linking protein, Fascin, then connects these filaments together into hexagonally packed bundles. We propose a similar mechanism of F-actin bundling in the Lepidoptera, given the patterns we observe for actin bundle formation as the scales elongate (Figs. 2 and 3).

Phalloidin staining shows the appearance of organized F-actin bundles at 21% of pupal development (36 h APF for *V. cardui*). At this point, the scale cells begin to project beyond the wing membrane as small bulbs (Fig. 2E–I). The rate of elongation is slow at first and increases over time as the scale cell increases in length. At 28% of pupal development (48 h APF for *V. cardui* and 72 h APF for *J. coenia*), the scale cells begin to elongate rapidly, and phalloidin staining indicates that this correlates with a rapid reorganization of the actin cytoskeleton into bundles located just under the membrane (Fig. 3). Note that most of the actin filaments at this stage run along the proximal–distal axis of the scale cell all the way to the tip, but the intensity of the phalloidin staining in Fig. 3 is weak toward the distal end of the cell, and WGA staining is more intense toward the distal tip.

Patterning of F-actin within the developing wing scale

As the scale elongates over time, we see a general trend that the actin bundles become thicker, but fewer in number (compare Fig. 3A'and B'-C' and D'; see also Supplementary Movies 1-4). We also find that there are two types of actin bundles within wing

scales (Fig. 3C-C" and D-D", see also Supplementary Movie 5): circumferentially-arranged peripheral actin bundles located just under the cell membrane and running the length of the scale, and internal actin bundles that do not extend to the distal tip, but are instead confined to the lower surface of the scale cell. In addition, the internal actin bundles only extend about 1/2-2/3 of the length of the scale, and continue proximally into the cell body (Fig. 3C–G). In Drosophila bristles, Bitan et al. (2012) report that actin bundles are absent at the bristle tip (where dynamic microtubules are present). We see that at 28% of pupal development (48 h APF for V. *cardui* and 72 h APF for *I. coenia*), most of the peripheral actin bundles reach close to the scale tip, but the intensity of phalloidin staining is weaker as they extend distally. By 31% of pupal development (53 h APF for V. cardui and 80 h APF for J. coenia), the peripheral actin bundles reach to the tip of the scale, but the internal actin bundles do not; indeed, we have never observed these internal bundles at the tip of the scale. Thus, some aspects of the dynamics of the actin bundles are similar to those seen in Drosophila, but others are different. The increased concentration of F-actin on the lower surface of the elongating scale cell is true for Drosophila bristles and hairs as well. Tilney and DeRosier (2005) find that the axial actin bundles in Drosophila bristles, which lie along the membrane, are larger on the inside of the bristle curve. Ghiradella (2010) has similarly observed that, in some lepidopteran species, the actin bundles on the underside of the wing scale cells are larger. We suggest that the internal cables on the lower side, plus the greater amount of actin in the peripheral cables on the lower side polarize developing wing scales (curving them distally, toward the wing edge).



Supplementary Movie 1. Scales of *Junonia coenia* 72 h APF – 3D projection. 3D projection and rotation of the same scales shown in Fig. 3A–A" and B–B". Developing wing scales at 28% of pupal development or 72 h APF, (developmental time is slightly longer for *J. coenia* than in *V. cardui*; this would be comparable to 48 h APF for *V. cardui*). WGA (magenta) stains cell membranes and phalloidin (yellow) stains F-actin. Scale cells have grown rapidly and now contain a multitude of fine actin bundles that are highly organized along the proximal–distal axis of the scale. For the actin bundles that do extend to the distal most end of the scale, the phalloidin staining intensity weakens toward the distal end of the scales, and WGA staining is more intense toward the distal tip doi:10.1016/j.ydbio.2014.06.005.



Supplementary Movie 2. Scales of *Junonia coenia* 72 h APF – planar sections. Planar sections through the same scales shown in Fig. 3A–A" and B–B". Developing wing scales at 28% of pupal development (72 h APF, developmental time is slightly longer for *J. coenia* than in *V. cardui*; this would be comparable to 48 h APF for *V. cardui*). WGA (magenta) stains cell membranes and phalloidin (yellow) stains F-actin. Movie begins with a 3D projection that rotates and switches to an angled planar view. Cross-sections are then shown, first in xy planes moving through different z positions, and then in xz planes moving through y positions, which provides cross-sectional views along the length of the scales. The xz planes first show both WGA and phalloidin staining, and then just WGA, and then just phalloidin. The movie ends by further rotating the planar projection and then returning to a 3D projection view doi:10.1016/j.ydbio.2014.06.005.

The formation of longitudinal ridges on central scale cells

In V. cardui, at around 43% of pupal development (72 h APF for V. cardui), WGA staining reveals just barely discernable longitudinal lines running along the length of the scales in the central portion of the wing (Fig. 4A). We think these may be the start of membrane pleats between the actin bundles (Fig. 4A' and A"). By 57% of pupal development (96 h APF for V. cardui), however, chitinous ridges have clearly formed between actin bundles (Fig. 4B-B", C-C"). Many scales possess actin bundles around their entire periphery, but the ridges on the underside of the scale are thinner than the ridges on the upper side of the scales. It may be that the larger, and more actin rich, bundles on the lower side of the scales inhibit ridge formation by inhibiting secretion. Preliminary observations suggest that wing scale width and length are directly correlated with the number of longitudinal ridges on the upper surface of the wing scale (unpublished results). Superresolution microscopy at this stage reveals that the individual actin bundles are up to 700 nm wide and tend to taper as they extend.

The formation of longitudinal ridges on edge scale cells

So far we have focused on the central scales that make up the majority of the scales of the wing, but it is worth noting some of the properties of the scales that are at the edge of the wings. These edge scales are not flattened, but instead remain quite cylindrical, and they extend out faster, and end up longer, than central scales. Edge scales have no detectable asymmetry in actin bundles, in keeping with their cylindrical nature. So the size of actin bundles,



Supplementary Movie 3. Scales of *Junonia coenia* 80 h APF – 3D projection. 3D projection and rotation of movie of the same scales shown in Fig. 3C–C^{*}. Developing *J. coenia* wing scale cells at 32% of pupal development (80 h APF; equivalent to 54 h APF in *V. cardui*) stained for WGA (magenta; cell membrane) and phalloidin (yellow, F-actin). Note that in this region the cover scales are far more elongated than the underlying ground scales. Scales have continued to elongate, and the actin bundles have become further organized into thicker bundles. Peripheral actin bundles have decreased in number, but increased in diameter, so that now individual bundles appear as distinct "rods" within the scale. Bundles on the underside of the scale are more intensely stained by phalloidin. In addition, approximately 6–12 "rods" are located internal to the cell membrane, but only on the lower surface of the scale. These internal bundles only extend about 2/3rds of the way along the proximal–distal axis and do not reach the distal end of the scale doi:10.1016/j.ydbio.2014.06.005.

and intensity of phalloidin staining of these bundles, is uniform around the circumference of an edge scale. Furthermore, edge scales have only peripheral actin bundles, with no internal actin bundles (Fig. 5; see also Supplementary Movie 6).

Ridges also form earlier in edge scales that in central scales. At 43% of pupal development (72 h APF for *V. cardui*), prominent ridges of cuticle can already be seen between the actin bundles (Fig. 5A–A" and B–B"; see also Supplementary Movie 7), and some of these ridges even have visible microstructures at their surfaces. We have also observed a small amount of F-actin just underneath the ridges (Fig. 5B'), but F-actin is never seen in this location before the ridges form. By 57% of pupal development (96 h APF in *V. cardui*), crossribs can be seen forming between ridges (Fig. 5C), and at this stage the prominent actin bundles are still seen between ridges, but there is an increasing accumulation of F-actin in locations underneath the ridges (Fig. 5C').

The disappearance of actin bundles begins at approximately 64% of pupal development (108 h APF). Breakdown of the bundles proceeds by two processes: the longitudinal splitting of bundles into sub-bundles and the disassembly of individual fragments. Very quickly all of the bundles are broken apart (Fig. 6A–A'), but by 71% of pupal development (120 h APF), actin bundle fragments continue to persist underneath the ridges (Fig. 6B–B'). Over the next few hours, the last of these bundles are degraded, and the scales no longer contain bundles of F-actin.

We were able to analyze a large number of pupae of *V. cardui* and *J. coenia* and generate a timeline of relatively closely spaced stages, and we saw essentially the same set of patterns of actin dynamics in both species. To see if our observations hold for



Supplementary Movie 4. Scales of *Junonia coenia* 80 h APF – more basal sections reveal relative positions of scale and socket cells and nuclei. Same scales as shown in Fig. 3C–C" and Supplementary Movie 3, but using z sections that are more basal (beginning at the lower portion of the scales and extending down into the layer of nuclei within the wing epithelium). The movie begins with a 3D projection that rotates and transitions to an xy planar projection. The xy planar projection then moves through various z positions, eventually reaching the level of the socket and scale nucleus. The projection then rotates, moves through z sections back to the lower part of the scales, and then returns to a 3D projection view. WGA in purple (cell membrane), phalloidin in yellow (F-actin), and DAPI in blue (nuclei) doi:10.1016/j.ydbio.2014.06.005.



Supplementary Movie 5. Scales of *Junonia coenia* 80 h APF – cross-section view. Rotating 3D projection of scales shown in Fig. 3D–D". *J. coenia* wing scale cells at 32% of pupal development (80 h APF; equivalent to 54 h APF in V. cardui) stained for WGA (magenta; cell membrane) and phalloidin (yellow, F-actin). Peripheral actin bundles appear as distinct "rods" just under the cell membrane, with bundles on the underside of the scale more intensely stained by phalloidin. Again, approximately 6–12 "rods" are located internal to the cell membrane, but only on the lower surface of the scale doi:10.1016/j.ydbio.2014.06.005.

other Lepidoptera, we obtained smaller numbers of pupae from several additional species, including *P. rapae*, *P. arcas/iphadamas*, *M. peleides*, *H. charithonia*, *A. vanillae*, and the moth *G. mellonella*.



Fig. 4. Ridge formation between actin bundles in central wing scales (*V. cardui*). WGA continues to stain the cell membrane of the scale, but also intensely stains forming cuticle and thus highlights the developing ridges and other surface substructures of the scales. The scales shown here are between the M2–M3 veins, but not at the edge of the wing (see Fig. 5) and represent the scales typical of the vast majority of the wing. (A– A^*) 43% of pupal development (72 h APF). Scales have flattened, and the finger-like projections at the ends of the scales are just forming. F-actin bundles (yellow) extend all the way to the distal end of the scales, and ridges are just beginning to appear on the surface of the scales. Scale bar=15 μ m. (B–B^{*}) 57% of pupal development (96 h APF). Scales have evy prominent ridges, and these ridges have formed between the existing actin bundles. When viewed from above, actin bundles can look irregular if the z focal planes extend through the entire scale, and the actin bundles are seen on both the upperside and underside (that is the case for the scale indicated with the arrow). Alternatively, if the focal planes extend through just part of the scale (as is the case for the scale indicated with the arrow). Alternatively, if the focal plane settend through just part of the scale (as is the case for the scale indicated with the arrow had), then the actin bundles can look incomplete as the scales tend to have some curvature. The precise relationship between the actin bundles are on both the scales are viewed in cross-section in C–C^{*}. Scale bar=15 μ m. (C–C^{*}) xz section through the scale at the level marked with the x–x line shown in B^{*}. The cross-section shows that the ridges are most prominent on the upper surface of the scale (facing away from the wing surface, see arrows in C). The actin bundles, and that there is no regular relationship between actin bundles). Merged image in C^{*} shows that the ridges have formed between actin bundles, and that there is no regular r

This provides us with a fair distribution within butterflies, and one moth as an outgroup. Our observations suggest that the various aspects of actin dynamics we have characterized in *V. cardui* and *J. coenia* are well conserved in other Lepidoptera. For example, in *A. vanillae* and *G. mellonella* we see the same arrangement of scale and socket cells as in *V. cardui* and *J. coenia* (Fig. 7A–A"' and G). We also observe the asymmetry of peripheral actin bundles on the upper and lower scale surfaces in all species examined (Fig. 7B–B' and F–F"), and the presence of internal actin bundles near the lower surface in *P. rapae* and *G. mellonella*. Ridges form between actin bundles in all these species, and then the actin is eventually degraded, but persists longer underneath the ridges (Fig. 7E–E").

In looking at these various species, we did find one exceptional pattern, and that was at later stages of pupal development in *A. vanillae*. Adult *A. vanillae* are mostly orange, black, and brown, but on the ventral surfaces of their wings, they also possess striking silver patches of scales (Fig. 8A, B). We observed that these silver scales had a distinctly different amount and

distribution of F-actin inside of them compared to any of the cells destined to be pigmented scales. Starting as early as 32% of pupal development (85 h APF), staining with phalloidin reveals a pattern that perfectly matches the adult pattern of silver scales. The distinction becomes even more striking by 43% of pupal development (113 h APF) (Fig. 8C). In none of the other species examined have we observed a pattern that prefigures any particular color pattern. The future silver scales contain far more F-actin than the scales that will have pigment colors. (Fig. 8D) and we confirmed that this appearance was not the result of simply having a greater concentration of scales in the silver regions (Fig. 8D'). As the scales flatten, we see that these silver scales contain double bundles of actin between each ridge (Fig. 8E, E'), possibly due to the regular alignment of actin bundles from the top and bottom surface. The other striking feature of these pupal silver scales is how flat and parallel they sit relative to one another when compared to adjacent scales that will be pigmented (Fig. 8F; see also Supplementary Movie 8). This flat and parallel orientation is also



Fig. 5. Ridge formation between actin bundles in edge wing scales (*V. cardui*). The scales at the edge of the wing are more bristle-like in that they are rounder and less flattened. These scales grow faster that central scales and become longer. They also lack asymmetry in the pattern of actin bundles; peripheral bundles are uniformly stained by phalloidin, and there is no sign of any internal bundles (see also Supplementary Movie 6). (A- A^{*}) 43% of pupal development (72 h APF). WGA staining reveals the prominent ridges, and these ridges have formed between each actin bundle. Scale bar=15 μ m (see also Supplementary Movie 7). (B-B") Cross-section (xz) of a scale shown in A-A". Arrowhead in B points at one ridge, same position indicated by arrows in B' and B". The ridges are positioned between the prominent actin bundles (arrow in B' and B"), but at this stage, a small amount of F-actin is also seen directly under the ridge (see arrowhead in B'). Our results indicate that the ridges form first, and only later does some F-actin appear under the ridges. Scale bar=5 μ m. (C-C") 57% of pupal development (96 h APF). WGA staining reveals that cross-ribs are developing between the ridges (arrow in C, same position indicated by arrows in C' and C") over the position of the prominent actin bundles. At this time, increased levels of F-actin are seen underneath the ridges (arrowhead in C', with same position shown by arrowhead in C and C"). Scale bar=5 μ m.



Supplementary Movie 6. Scales of *Vanessa cardui* 50 h APF. 3D projection movie of isolated scales from the edge of the wing stained for WGA (magenta; cell membrane) and phalloidin (yellow, F-actin). Compared to non-edge scales, edge scales contain no internal actin bundles, show no obvious asymmetry in the intensity of phalloidin staining, and are much rounder. Like all other scales, however, the ridges (shown here in purple) form between the actin bundles (yellow) doi:10.1016/j.ydbio.2014.06.005.

seen for the adult silver scales (Fig. 8G). This orientation of the scales contributes to scale iridescence and the silvery, almost mirrored finish of these areas of the wing. This was found to be true in the closely related silverspot butterfly, *Dione juno*, where it was noted that increased parallel stacking created a larger reflectance than a single silver scale (Giraldo, 2008). Given that the actin



Supplementary Movie 7. Scales of *Vanessa cardui* 72 h APF. 3D projection movie of the same edge scales shown in Fig. 5A–A" and B–B" stained for WGA (magenta; cell membrane) and phalloidin (yellow, F-actin). At this time point, some edge scales have become a little flatter, although they never become as flat as non-edge scales. The ridges (shown here in purple) are located between the actin bundles (yellow) and project away from the surface of the scale doi:10.1016/j.ydbio.2014.06.005.

cables may regulate scale shape and orientation, we hypothesize that this increased F-actin in the silver scales is responsible for their flat and parallel orientation, and ultimately contributes to their iridescent properties.



Fig. 6. Actin degradation. (A–A") 64% of pupal development (108 h APF). F-actin bundles are rapidly broken down. The bundles appear to disintegrate and break into smaller pieces. Scale bar=15 µm. (B–B") By about 70% of pupal development (120 h APF) what little F-actin remains is concentrated under the ridges. Within the next 12 hours, even this actin will disappear and no detectable (above background) F-actin will remain within the scale. Scale bar=15 µm.



Fig. 7. Conservation of overall scale formation in Lepidoptera. General patterns of scale development, including patterns of F-actin localization, seem to be well conserved in Lepidoptera. (A-A") *Agraulis vanilla*e (Gulf Fritillary, Heliconiinae). A – WGA, A' – phalloidin, A" – DAPI, A" – merge. The initial orientation of the scale and socket nuclei (magenta and green arrows respectively in A") are oriented as in *V. cardui* and *J. coenia*, as are the initial patterns of F-actin and scale growth (A and A'). In A and A', arrows point to ground scales, and arrowheads point to cover scales, which alternate in position along each row. Note that the angle of the scales is no longer perpendicular to the rows of scale/socket nuclei, but this is due to shear force when the wing was coverslipped. Scale bar=15 µm. (B-E") *Pieris rapae* (Cabbage White, Pieridae). B–D and E' – phalloidin, E – WGA, E" merge of E and E'. (B) During the rapid phase of scale elongation (as seen in cross-section), actin bundles are positioned around the periphery of the scale, with more intense phalloidin staining seen on the lower surface of the scales (arrow). Scale bar=100 µm. (C) At a focal plane just above the socket, the actin bundles of the lower surface of the scale can be seen (arrow) as well as internal bundles (arrowhead), also located on the underside of the scales. Scale bar=10 µm. (D) At the level of the socket (arrowhead), the peripheral and internal bundles merge (arrow) and extend down into the wing epithelium. Scale bar= 10 µm. (E-E') As F-actin degrades, it first disappears from between the ridges, but remains for a while underneath the ridges. Scale bar=15 µm. (F–G) *Calleria mellonella* (Greater Wax Moth, Pyralidae). (F–F') F – WGA, F' – phalloidin, F'' – merge. Just as in butterfiles, moth scales show peripheral F-actin bundles with an asymmetry – more intense phalloidin staining in a subset of bundles on the lower surface of the scale, and docover scales. Scale bar=15 µm. (G) Cross-section through the wing surface, D



Fig. 8. Silver scales of the Gulf Fritillary (*Agraulis vanillae*). (A) Underside (ventral side) of adult butterfly. Scale bar = 1cm. (B) Pattern of silver spots on the ventral surface of the adult hindwing. Scale bar = 0.5 cm. (C) Phalloidin staining of a 32% of pupal development (113 APF) hindwing (ventral surface). At this time, scales are undergoing their phase of rapid elongation (equivalent to about 54 h APF for *V. cardui*). For all other species examined, the phalloidin staining would look rather uniform at this magnification, but here the pattern clearly resembles the future pattern of silver scales on the adult. Scale bar = 2 mm. (D and D') Higher magnification view of a boundary region between the two different levels of phalloidin staining. Red dots mark the boundary of sliver scales. Scale bar = 50 μ m. (D) Each scale in the future silver region (s) shows more intense phalloidin staining (and presumably greater F-actin concentration) than the scales in the pigmented region (p). (D') DAPI staining of the same region shows that there is no difference in scale density between the future silver (s) and pigmented (p) regions. (E and E') Phalloidin (yellow) and WGA (magenta) staining of silver scale. Pairs of F-actin bundles are seen between each ridge, as opposed to the single actin bundle seen between ridges in other species. Scale bar = 2 μ m. (F) 3D view of WGA stained wing (140 APF), tilted to an angle to show that the future silver scales (s side) are far flatter and parallel to one another compared to the scales on the future pigmented (p) side. Scale bar = 200 μ m (see also Supplementary Movie 8). (G) SEM view of adult scales. Darker looking scales are pigmented scales, while the lighter scales are the silver scales. Again, the silver scales appear far more organized and parallel to one another. Scale bar = 100 μ m. (H) High magnification SEM view of pigmented and silver scale surfaces. The pigmented scale (p) shows a typical cross-rib pattern between ridges, but the silver scale (s) shows



Supplementary Movie 8. Boundary between silver and pigmented scales of *Agraulis vanillae* 140 h APF. 3D projection movie of the same scales shown in Fig. 8F stained for WGA (magenta; cell membrane) and phalloidin (yellow, F-actin). The future silver scales (right side) are far more organized, as well as flatter and parallel to one another, compared to the scales on the future pigmented (left) side. In addition, there is a higher concentration of F-actin in the future silver scales compared to the future pigmented scales doi:10.1016/j.ydbio.2014.06.005.

All of our observations of F-actin dynamics in the various lepidopteran species examined, along with the understanding that has come from the genetic and molecular analysis of bristle development in *Drosophila*, allow us to hypothesize various roles for F-actin in shaping lepidopteran scales. While the tools are not yet available to easily carry out a genetic approach to butterfly scale development, we were able to use cytochalasin-D to disrupt actin dynamics specifically at different time points to test our hypotheses regarding scale development.

Inhibiting actin polymerization aborts the elongation and ornamentation of wing scales

Cytochalasin D, an actin assembly inhibitor, in low concentrations caps the barbed end of actin filaments and inhibits further elongation from the barbed end (Cooper, 1987). In our study, pupal wing pouches were injected at various time points with cytochalsin D and were allowed to grow until the point when wing scale development would be complete (approximately 96 h APF/57% of pupal development), at least as far as overall growth and topology are concerned. We show that cytochalasin D prevents the elongation of scale cells during early stages of their development (Figs. 9 and 10 "48 h"). Our results indicate that in the presence of cytochalasin D scale cell elongation was



Fig. 9. Treatment with cytochalasin D at 24 h APF. In pupae injected at 24 h after pupation, actin filaments within cells of the wing epithelium are disrupted and scales do not form and elongate. Note that at this early stage, Wheat Germ Agglutinin stains the nuclear envelope (white arrows in A). (A–A") Control pupae were injected with DMSO at 24 h APF and dissected 2 h after injection (26 h APF). Other pupae were injected with Cyto-D at 24 h APF, and dissected 2 h after injection (28 h APF, B–B".) or 96 h APF (C–C"). At our rearing temperatures, the scale cells of *Vanessa cardui* are fully formed by 96 h APF (D–D"). Purple=WGA (cell membrane/chitin marker); yellow is phalloidin. Scale bar=16 μ m.

dramatically slowed, while the controls elongated normally. Depolymerization of actin bundles early in development prevents the elongation of the scale. Treatment later in development disrupts regular "tiling" of scales and disrupts longitudinal ridge formation but does not collapse scale cells (Figs. 10 and 11). It is likely that the actin filaments are forming thicker, cross-linked bundles at this point. In addition, the bundles likely halt turnover at the point of rapid elongation (if there is turnover of the filaments they will be unable to reassemble and will gradually disappear) (Tilney et al., 2000). Applying Cytochalasin D to the pupa at around 32% of pupal development (54 h) disrupts longitudinal ridge formation, and the scale cells do not lay flat to the membrane (Fig. 10 "54 h"). The loss of ridges by treatment at this time point may reflect the requirement of actin turnover to space the actin bundles properly and allow ridge formation to begin. Very late disruption does not collapse scales, but does lead to the loss of most distal patterns (Fig. 11). We observe that finger-like

projections along the distal edge of the cell do not appear, suggesting that F-actin is necessary for their formation. However, the mechanisms involved in their number and positioning are unknown.

In summary, inhibiting actin polymerization by treating scale cells with cytochalasin D can abort the development of certain scale properties. We find that F-actin is required to elongate butterfly wing scale cells, and to position longitudinal ridges (chitin secreted between bundles). F-actin is also necessary for the development of finger-like projections at the tip of scale cells (as treatments with cytochalasin D can inhibit their formation). After about 57% of pupal development (96 h APF in *V. cardui*), cytochalasin-D treatment has no effect on scale morphology, suggesting F-actin is not necessary for later stages of wing scale development. These results are consistent with experiments that were performed on *Drosphila* thoracic bristles by Tilney et al. (2000).



Fig. 10. Treatments with cytochalasin D, at 48 h and 54 h APF. In scales injected at 48 h after pupation, actin filaments do not fully break apart, but scale cells appear to collapse. (A-A") Control pupae were injected with DMSO at 48 h APF and dissected 2 h after injection (50 h APF). Other pupae were injected with Cyto-D at 48 h APF, and dissected at 2 h after injection (50 h APF, B–B") or 96 h APF (C–C"). In scale cells injected at 54 h after pupation, actin filaments do not break down completely, and scales do not collapse, but finger-like projections and longitudinal ridges do not develop. Actin filaments of injected animals do not depolymerize after 54 h APF, which indicates that actin-turnover and filament cross-linking cease after this point. D–D" Control pupae were injected with DMSO at 54 h APF and dissected 2 h after injection (56 h APF). Other pupae were injected with Cyto-D at 54 h APF, and dissected at 2 h after injection (56 h APF, E-E") or 96 h APF (F-F"). Purple=WGA (cell membrane/chitin marker); yellow is phalloidin. Scale bar = 16 µm.



WGA Phalloidin Overlay

injection 96 hr post-pupation



Fig. 11. Treatments with cytochalasin D, at 66 h and 96 h APF. In scale cells injected at 66 h after pupation, finger-like projections and longitudinal ridges do not develop. (A-A") Control pupae were injected with DMSO at 66 h APF and dissected 2 h after injection (68 h APF). Other pupae were injected with Cyto-D at 66 h APF, and dissected at 2 h after injection (68 h APF, B-B") or 96 h APF (C-C"). In scale cells injected at 96 h after pupation, injection cytochalasin D does not alter scale cell morphology (at this point, scale cells have fully developed). (D-D") Control pupae were injected with DMSO at 96 h APF and dissected 2 h after injection (98 h APF). Other pupae were injected with Cyto-D at 96 h APF, and dissected at 2 h after injection (98 h APF, E-E"). Purple=WGA (cell membrane/chitin marker); yellow is phalloidin. Scale bar=16 µm.

Conclusion

The outlines and surface sculpture of butterfly wing scales vary widely, and this architectural complexity arises from variations in the developmental program of the individual scale cells of the wing epithelium. To begin addressing the cellular basis of the formation of a wing scale, we performed the first F-actin inhibition experiments on butterfly wing scales. We demonstrate that F-actin

plays a major role in wing scale elongation, and that the arrangement of actin bundles produces many of the morphological features seen in butterfly wing scales. Our results show that (1) F-actin is needed for initial scale cell elongation, (2) F-actin is needed to orient the scale cell parallel with the wing membrane, (3) F-actin plays an essential role in the positioning of longitudinal ridges (where most microstructures are located), and (4) F-actin is necessary to produce finger-like projections at the tips of wing scales. This study establishes an outline of how a wing scale develops and creates the intricate cuticular structures that pattern its surface. In order to further this knowledge, future studies should examine the influence of additional cellular processes, such as membrane folding, on the development of other wing scale structures, including photonic crystals.

Acknowledgments

We thank the following for providing us with lab space for portions of this work: The Embryology Course at the Marine Biological Laboratory at Woods Hole; Ryan Fessenden of the University of Florida McGuire Center for Lepidoptera and Biodiversity; Daniel Hahn, University of Florida; and Fred Gagnon of Magic Wings Butterfly Conservatory and Gardens. We also wish to thank Ulf Schwarz, Christian May, and David Castaneda-Castellanos of Leica Microsystems for providing us access to the super-resolution microscopes and Rob White for the FP6.87 monoclonal. This research was supported by Yale University, the University of California, Berkeley, and an NSF GRFP awarded to A. Dinwiddie.

References

- Beldade, P., Koops, K., Brakefield, P.M., 2002. Developmental constraints versus flexibility in morphological evolution. Nature 416, 844–847.
- Bitan, A., Guild, G.M., Bar-Dubin, D., Abdu, U., 2010. Asymmetric microtubule function is an essential requirement for polarized organization of the Drosophila bristle. Mol. Cell Biol. 30 (2), 496–507.
- Bitan, A., Rosenbaum, I., Abdu, U., 2012. Stable and dynamic microtubules coordinately determine and maintain Drosophila bristle shape. Development 139 (11), 1987–1996.
- Carroll, S.B., Gates, J., Keys, D.N., Paddock, S.W., Panganiban, G.E., Selegue, J.E., Williams, J.A., 1994. Pattern formation and eyespot determination in butterfly wings. Science 265 (5168), 109–114.
- Cho, E.H., Nijhout, H.F., 2013. Development of polyploidy of scale-building cells in the wings of *Manduca sexta*. Arthropod Struct. Dev. 42 (1), 37–46.
- Colombani, J., Bianchini, L., Layalle, S., Pondeville, E., Dauphin-Villemant, C., Antoniewski, C., Carre, C., Noselli, S., Leopold, P., 2005. Antagonistic actions of ecdysone and insulins determine final size in drosophila. Science 310 (5748), 667–670.
- Cooper, J.A., 1987. Affects of cytochalasin and phalloidin on actin. J. Cell Biol. 105, 1473–1478.
- Farnesi, L.C., Brito, J.M., Linss, J.G., Pelajo-Machado, M., Valle, D., Rezende, G.L., 2012. Physiological and morphological aspects of *Aedes aegypti* developing larvae: effects of the chitin synthesis inhibitor novaluron. PLoS ONE 7 (1), e30363.
- Galant, R., Skeath, J.B., Paddock, S., Lewis, D.L., Carroll, S.B., 1998. Expression pattern of a butterfly achaete-scute homolog reveals the homology of butterfly wing scales and insect sensory bristles. Curr. Biol. 8 (14), 807–813.
- Ghiradella, H., 1998. Hairs, bristles and scales. In: Locke, M. (Ed.). Microscopic Anatomy of Invertebrates., vol. 11A. Wiley-Liss, New York, USA, pp. 257–287 (Insecta).
- Ghiradella, H., 2010. Chapter 4 Insect cuticular surface modifications: scales and other structural formations. In: Jérôme, C., Stephen, J.S. (Eds.), Advances in Insect Physiology, vol. 38. Academic Press, pp. 135–180.

- Giraldo, M.A., 2008. Butterfly Wing Scales: Pigmental and Structural Properties (Ph.D. thesis). University of Groningen.
- Greenstein, M., 1972. The ultrastructure of developing wings in the giant silk moth Hyalophora cecropia. II. Scale-forming and socket-forming cells. J. Morphol. 136, 23–52.
- Guild, G.M., Connelly, P.S., Ruggiero, L., Vranich, K.A., Tilney, L.G., 2005. Actin filament bundles in Drosophila wing hairs: hairs and bristles use different strategies for assembly. Mol. Biol. Cell 16 (8), 3620–3631.
- Held, L.I.J., 2002. Imaginal Discs: The Genetic and Cellular Logic of Pattern Formation. Cambridge University Press, Cambridge, UK.
- Joron, M., Frezal, L., Jones, R.T., Chamberlain, N.L., Lee, S.F., Haag, C.R., Whibley, A., Becuwe, M., Baxter, S.W., Ferguson, L., Wilkinson, P.A., Salazar, C., Davidson, C., Clark, R., Quail, M.A., Beasley, H., Glithero, R., Lloyd, C., Sims, S., Jones, M.C., Rogers, J., Jiggins, C.D., ffrench-Constant, R.H., 2011. Chromosomal rearrangements maintain a polymorphic supergene controlling butterfly mimicry. Nature 477 (7363), 203–206.
- Kelsh, R., Weinzieri, R.O.J., White, R.A.H., Akam, M., 1994. Homeotic gene expression in the locust *Schistocerca*: an antibody that detects conserved epitopes in *Ultrabithorax* and *Abdominal-A* proteins. Dev. Genet. 15, 19–31.
- Kolle, M., Salgard-Cunha, P.M., Scherer, M.R.J., Huang, F., Vukusic, P., Mahajan, S., Baumberg, J.J., Steiner, U., 2010. Mimicking the colourful wing scale structure of the *Papilio blumei* butterfly. Nat. Nano 5 (7), 511–515.
- Lees, A.D., Picken, L.E.R., 1945. Shape in relation to fine structure in the bristles of Drosophila melanogaster. Proc. R. Soc. Lond. Ser. B, Biol. Sci. 132 (869), 396–423.
- Locke, M., 1967. The developmental patterns in the integument of insects. In: Brachet, M.A.A.J. (Ed.), Advances in Morphogenesis, vol. 6. Academic Press, New York, pp. 33–88.
- Martin, A., Papa, R., Nadeau, N.J., Hill, R.I., Counterman, B.A., Halder, G., Jiggins, C.D., Kronfrost, M.R., Long, A.D., McMillan, W.O., Reed, R.D., 2012. Diversification of complex butterfly wing patterns by repeated regulatory evolution of *Wnt* ligand. Proc. Natl. Acad. Sci. 109 (31), 12632–12637.
- Merzendorfer, H., Zimoch, L., 2003. Chitin metabolism in insects: structure, function and regulation of chitin synthases and chitinases. J. Exp. Biol. 206 (Part 24), 4393–4412.
- Nijhout, H.F., 1991. The Development and Evolution of Butterfly Wing Patterns. Smithsonian Institution Press, Washington D.C.
- Picken, L.E.R., 1949. Shape and Molecular Orientation in Lepidopteran Scales. Philos. Trans. R. Soc. Lond. Ser. B, Biol. Sci. 234 (608), 1–28.
- Reed, R.D., Papa, R., Martin, A., Hines, H.M., Counterman, B.A., Pardo-Diaz, C., Jiggins, C.D., Chamberlain, N.L., Kronforst, M.R., Chen, R., Halder, G., Nijhout, H.F., McMillan, W.O., 2011. Optix drives the repeated convergent evolution of butterfly wing pattern mimicry. Science 333 (6046), 1137–1141.
- Roegiers, F., Younger-Shepherd, S., Jan, L.Y., Jan, Y.N., 2001. Two types of asymmetric divisions in the Drosophila sensory organ precursor cell lineage. Natl. Cell Biol. 3 (1), 58–67.
- Saranathan, V., Osuji, C.O., Mochrie, S.G.J., Noh, H., Narayanan, S., Sandy, A., Dufresne, E.R., Prum, R.O., 2010. Structure, function, and self-assembly of single network gyroid (I4132) photonic crystals in butterfly wing scales. Proc. Natl. Acad. Sci. 107, 11676–11681.
- Simonsen, T.J., Kristensen, N.P., 2003. Scale length/wing length correlation in Lepidoptera (Insecta). J. Nat. Hist. 37 (6), 673–679.
- Stavenga, D.G., Stowe, S., Siebke, K., Zeil, J., Arikawa, K., 2004. Butterfly wing colours: scale beads make white pierid wings brighter. Proc.: Biol. Sci. 271 (1548), 1577–1584.
- Stoehr, A.M., Walker, J.F., Monteiro, A., 2013. Spalt expression and the development of melanic color patterns in pierid butterflies. EvoDevo 4 (1), 6.
- Tilney, L.G., Connelly, P.S., Vranich, K.A., Shaw, M.K., Guild, G.M., 2000. Actin filaments and microtubules play different roles during bristle elongation in Drosophila. J. Cell Sci. 113 (Part 7), 1255–1265.
- Tilney, L.G., DeRosier, D.J., 2005. How to make a curved Drosophila bristle using straight actin bundles. Proc. Natl. Acad. Sci. 102 (52), 18785–18792.
- Tilney, L.G., Tilney, M.S., Guild, G.M., 1995. F actin bundles in Drosophila bristles. I. Two filament cross-links are involved in bundling. J. Cell Biol. 130 (3), 629–638.
- Turner, C.M., Adler, P.N., 1998. Distinct roles for the actin and microtubule cytoskeletons in the morphogenesis of epidermal hairs during wing develop-
- ment in *Drosophila*. Mech. Dev. 70 (1-2), 181–192. Vukusic, P., Sambles, J.R., Ghiradella., H., 2000. Optical classification of microstruc-
- ture in butterfly wing-scales. Photon. Sci. News, 61–66.
- Vukusic, P., Sambles, J.R., Lawrence, C.R., Wootton, R.J., 1999. Quantified interference and diffraction in single Morpho butterfly scales. Proc. R. Soc. Lond. Ser. B: Biol. Sci. 266 (1427), 1403–1411.