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Cytoskeletal Organization, its Evolution and Development, in Amphiesmenopteran Macrochaetae

By

Kyle Ambrose DeMarr

A dissertation submitted in partial satisfaction of the
requirements for the degree of
Doctor of Philosophy
in
Integrative Biology
in the
Graduate Division
of the
University of California, Berkeley

Committee in charge:
Professor Nipam H. Patel, Co-chair
Professor Charles R. Marshall, Co-chair
Professor Peter H. Sudmant
Professor Iswar K. Hariharan

Spring 2024

Abstract

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in Amphiesmenopteran Macrochaetae

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Doctor of Philosophy in Integrative Biology

University of California, Berkeley

Professor Nipam H. Patel, Co-chair

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This dissertation aims to bring new perspective to our understanding of the growth, development, and evolution of the vestiture which adorns the wings of the Lepidoptera—butterflies and moths. This perspective is wrought from both advancing imaging techniques to observe the growth of these elaborate cell-secreted structures directly and through examining parallel trends in the scales and bristles of their sister order, the Trichoptera—caddisflies.

Firstly, I develop methods to observe at high resolution the organization, arrangement, and dynamics of the cytoskeleton and cellular organelles through early scale development using commercially available live cell dyes. These protocols permit observing any timepoint of wing growth for any butterfly and moth hindwing, and any wing surfaces of insect species with transparent pupal cuticle. These techniques help confirm that scales grow through actin filament elongation and subsequent bundling into thick rods and allow us to observe nuclear divisions and lineages of sensory organ precursors across species. Secondly, I correlate patterns of microtubule and actin filament distribution across scale types throughout the lepidopteran phylogeny and make an association between reticulated microtubule networks and flattened scale morphologies. I then identify a transcription factor that seems to specify a cylindrical, non-flattened, scale shape fate and tie this into the evolution of transparency and specialized, elongated androconial scales. Using live-imaging of microtubule networks across normal and drug-perturbed development, I connect these cytoskeletal and transcription factor data to hypothesize that this gene initiates a cellular program to stabilize microtubules, preventing their transition to a reticulate network and producing cylindrical cell shapes and that this fate switch has been used in the evolution of transparent wing patches and scent-dispersing scales throughout lepidopteran evolution. Lastly, I develop tools, including a genome, to study a genus of long-horned caddisflies, *Nectopsyche*, that has independently evolved scales resembling those of butterflies and moths, and present phylogenetic and morphological data to contextualize this convergence with our current knowledge of macrochaete cytoskeletal organization and the evolution of wing patterns.

Altogether, this document establishes a first step in producing an understanding of comparative growth dynamics of amphiesmenopteran macrochaetae with an eye to uncover general trends linking cytoskeletal structure and cellular morphology.

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Thesis Overview

This dissertation aims to bring new perspective to our understanding of the growth, development, and evolution of the vestiture which adorns the wings of the Lepidoptera—butterflies and moths. This perspective is wrought from both advancing imaging techniques to observe the growth of these elaborate cell-secreted structures directly and through examining parallel trends in the scales and bristles of their closest relatives, the caddisflies of the order Trichoptera. Altogether, this document establishes the first step in producing an understanding of comparative growth dynamics of amphiesmenopteran macrochaetae with an eye to uncover general trends linking cytoskeletal organization and cell morphology.

As an introduction to the dissertation, I recontextualize current thinking of structural relationships in taxonomy and evolution with developmental insights before explicating how this new perspective links cellular dynamics to imaginal morphology and ecology, working toward a proposition to view macrochaete evolution in a morphospace framework.

In chapter I, I outline a method to observe living dynamics of the cytoskeleton and cellular organelles of scale and bristle cells in the hindwings of any lepidopteran and in both wings of any insect with transparent pupae using confocal and super-resolution microscopy. I push forward *Plutella xylostella*, the diamondback moth, as a captivating species in which to observe scale growth dynamics, both for its optical transparency and very short generation time. I enlist this method to confirm that actin filaments condense into rods through a co-linking/bundling process, as expected from *Drosophila* bristles, using fluorescence recovery after photobleaching experiments conducted across different regions of the growing scale, and directly observe the lineage of the sensory organ precursor cell that produces the scale and socket. Lastly, I demonstrate the potential in this method to observe other cellular phenomena including mitochondrial movements and vesicular transport. In a subsection, I further modify this protocol to accommodate species acquired as pupae and capture live actin dynamics in structurally colored green scales in the species *Papilio palinurus*, offering insight to how even seemingly well-conserved aspects of scale development like ridge formation can be modified in striking ways to produce structural coloration.

In chapter II, I home in on a pervasive heterogeneity in scale morphotype observed across Lepidoptera: long, bristle-like cylindrical scales, common along the wing edge, androconial patches, and in the evolution of transparent wing regions, and the more typical lamelliform, flattened scales which most know to imbricate upon the wing, giving rise to species-specific patterns. Through serial dissections and staining to compare the distribution of actin filaments and microtubules across six species that span this axis of scale variation and 120 million years of evolutionary time, I propose that linking of these cytoskeletal networks, especially in the early stages of scale budding and elongation, gives rise to more cylindrical scale morphologies. Going further, I pharmacologically manipulate microtubule stability in living scales to demonstrate that a higher degree of stability causes a more cylindrical growth pattern in developing scales. Using associations between gene expression patterns known from *Drosophila* and lepidopteran wing

edges, I identify a transcription factor protein, Cut, which localizes to the nuclei of interior scales with bristle-like morphotypes across species. I hypothesize that this factor activates a downstream cascade of expression that stabilizes microtubules in expressing scale cells to produce bristle-like macrochaetae. In a first step to identify these factors, I develop and describe a technique to dissociate lepidopteran wings while keeping full cells and nascent budding scale extensions intact to lay the groundwork for future transcriptional studies.

In chapter III, I investigate an example of convergent evolution in the caddisfly genus *Nectopsyche*, in which several lepidopterous species exist. To understand the polarity and pattern of this trait, I construct a phylogeny representing ~50% of species diversity and all major taxonomic groupings using an anchored hybrid enrichment probeset derived from the 1kITE initiative. Secondly, I sample wing macrochaetae from each represented species and map their structural traits to branches to demonstrate that lepidopteran characteristics converge in a punctuated manner within caddisfly scales, whereby some species possess a mix of, but not all, classical scale features. Overlaying wing pattern elements in a similar manner reveals either multiple independent origins of or several reversals from an ancestral jumping spider-mimic pattern that can be found in four independent species groups. Thirdly, I select a single species, *N. exquisita* to serve as a workhouse and produce a genome and developmental series for pupal wing growth spanning early scale elongation to full extension, focusing on cytoskeletal organization. I furthermore develop methods for egg collection from wild-caught adults and rearing of larvae independent of their preferred wild hostplant, setting the stage for future interrogation of wing pattern *via* CRISPR or other genetic manipulations.

Acknowledgements

Any PhD is an epic, and mine just felt a little more like Odysseus's than I would have liked. Yet, although my journey was nothing like how I envisioned or planned, I am thankful for the winding path having led me to meet and work with so many truly wonderful and generous people. Mentioned here are only a fraction, but to all who have helped me along this path, or just been on the receiving end of my rambling, I am grateful and thank you.

Any good trip starts at the beginning. To my parents, your sacrifices and guidance gave me the strongest foundation I could hope for in tackling the world. I would not be who I am today without your love and support for my early curiosities. To my sister, I am so impressed and happy for the life you have created and thank you for advocating for me and always being there to talk.

I have had many wonderful teachers as I discovered my love for evolutionary biology, but I want to particularly highlight some that I still think back to regularly for their steering influence. Ann Eichenmuller, for being an inspiration and icon, pushing me to appreciate the written word and go beyond what had been set in the curriculum, and indulging my love of macabre literature at an important stage of my life; Jessica Yohe, for offering a greater view of the world both with the labs you put together and in your advice and conversation; Amanda Myatt, for being an advocate for me and recommending me for opportunities to think and do science out of the classroom; and Cindy Young, for being a champion, always being there for support, and always being a riot and laugh at lunchtime.

I saw my first sliver of the bigger world in tiny Ithaca. My time at Cornell would have been much less rich if not for the professors, employees, and fellow students with whom I had the privilege of learning and growing into colleagues. Dr. Kerry Shaw, to this day you have given me some of the best life advice and I sincerely appreciate your candor in allowing me to develop my habits; it was a great first lab job and research experience and your classes were always illuminating. Dr. Bryan Danforth, your courses were everything I had ever dreamed of studying and doing as a kid and your enthusiasm was always palpable; thank you sincerely for always stepping in to help and make opportunities possible. Dr. Angela Douglas, Insect Physiology stands as the archetype for course syllabi. Your calculated instruction was as impressive to see as the experiments you drew out from the empirical beginnings of entomology. I developed a love for dissection and crazy experimental manipulations under your tutelage. Dr. Kelly Liu, you were my introduction to developmental biology which I took at a point when I was feeling dissatisfied with the limitations that kept coming up in evolutionary biology classes. There was no better subject to fill that hole in my heart and your class was the best and most organized I have ever taken. I marvel at the ground we covered and the clarity of your presentations that captured the beautiful logic in determining necessity and sufficiency.

Dr. Cole Gilbert, you are just a cool dude. Majoring in Entomology would not be the same, and not anywhere near as fun, without your presence. You taught me the foundations and I am

forever indebted and grateful; teaching with you and getting to see how to run a class were the best introductions to course instruction I could have gotten. Dr. Jason Dombroskie, bosses do not get any better than you—working in the insect collection will remain a highlight of my life and I always appreciated your guidance and pop quizzes for insect identification. Dr. Jim Liebherr, chatting with you in the doorway to your office in the collection about literally anything was always amazing and I cherish that we can still relive that to some extent at conference socials today. Teaching Larval with you and going out collecting was one of the bright spots in my final year. Dr. Bob Reed, thank you for allowing me to work in your lab as a senior; although short, it could not have prepared me better for graduate school. I can begrudgingly say that I have turned around to no longer hating Lepidoptera and can appreciate they are, in fact, sometimes cool (but mostly for their scales). Dr. Amy McCune, it is not a lie to say that I did not graduate early because I wanted to take Macroevolution. As extreme a choice as that was, I do not even come close to regretting it. The literature we read together as a class literally changed my brain and I owe my evolutionary perspective to your Devil's advocacy and Socratic method. Dr. Mariana Wolfner, you introduced me to EvoDevo which was everything I have ever wanted in biology. Hands-down the most enjoyable class that scratched an itch that I did not even know I had. Since I have graduated, you have remained an important mentor and valued friend, and I admire how you exemplify fantastic science while being a great human being. Lastly, it would be remiss to not mention my fellow Entomology majors, particularly Drs. Ryan St Laurent, Ben Lee, and Leah Buchmann, and the SnodWiggs, especially Jamie Freeman, Dan Pearlstein, and Elias Diakolios. Thank you for giving me the true college experience in every sense of the word and friendships that will continue for the rest of our lives.

I was very lucky to have had the opportunity to work in several entomological collections as an undergraduate and these experiences have deepened my love for the natural world in every way and given me colleagues for life. To my first ever mentor, Dr. Ollie Flint, Jr. you were a treasure that was always generous, kind, and a wealth of knowledge and great stories. I wish I could show you what I was able to do with the start you have given me, but I will always cherish my memories of you. Dr. Torsten Dikow, thank you for your support and friendship in the wake of Ollie's passing and your great help in continuing to work with the collection. Your research acumen and organization is only seconded by your kindness. Drs. Dave Grimaldi and Steve Davis, you got me hooked on insect scales and set my future research career into motion. Words cannot describe how much I am impressed by your collective entomological knowledge and research diligence. To Steve especially, working with you as a summer intern was some of the most fun I have had in a lab and I am so glad I can call you both a friend and colleague. To my cohort of American Museum of Natural History interns, you all are the best. It has been a joy watching you all succeed in your different life paths.

My time on the University of California, Berkeley itself was too short in time but not in great people. Thank you to my qualifying committee, Drs. Marvalee Wake, Seth Finnegan, Paul Fine, and Kip Will for taking the time to chat extensively about evolutionary biology for hours and giving great perspective on the state of the field. Dr. Britt Koskella, it was an honor to be a part of teaching your dream course for the first time. The conversations and advice as we walked to

and from teaching Coevolution were some of my favorite things from my time on campus. Dr. Pete Oboyski, thank you for letting me hang out in the insect collection to get my fix every now and again. The Essig collection's *Nectopsyche tapanti* specimen was the first I inspected closely in person that made me fall in love with the genus. My cohort were a great mix of talented and incisive researchers with whom I felt lucky to start my journey into independent research. Dan Wait and Dr. Jesús Martínez-Gómez, you especially made my time brighter and it is always great when we can connect again, however briefly. Monica Albe, conducting PhD research remotely across the country would have been near impossible without your guidance and you keeping on top of all the minutiae. Tremendously grateful for your help and support.

To Drs. Elisa Visher and Lewis Bartlett, you are my support network and I am so lucky to have you as my crew. No matter where we end up, I know that we will continue to lean on each other for advice, a new perspective, or just words of support. Your approaches to science, that being the practice or the industry, motivate and inspire me beyond words and I know that you change academia for the better. Lewis, I am so happy to see your career progress and know that you are on your way to becoming a titan of your field. How you mix theory and empiricism in real-world apiculture should be a model for how others devise their science and motivates me to pursue basic science avenues in my own work that have the highest chance of bettering the world. Elisa, there are no words for you but superstar. The already high bar you hold for yourself somehow keeps getting surpassed with ease and no sign of waning. I am in awe of your actualization and aim to emulate a fraction of your organization. Your ability to dissect questions to the theoretical core will take you to wherever you want to go, and I hope to visit as much as I can manage.

To my dissertation committee, Drs. Peter Sudmant, Charles Marshall, and Iswar Hariharan, thank you for always being there to give a thumbs-up and words of guidance, and extra thanks to Charles for becoming the Berkeley-based co-chair after the lab moved and hooding me at commencement.

For better or worse, the Marine Biological Laboratory became my home and I would not have been able to make it through without the wonderful people that make it tick. Dr. Abhishek Kumar, your lab became my second home and the core of my social life at the MBL. You are a great friend, mentor, and human who I am lucky to have met and continue to work with, despite my difficult samples. Dr. Matthew Parent, Anthony Mautino, and Will Ramos, conversations with you all, although tangential, got me through an innumerable number of tough weeks, thank you for your friendship and generosity in time when it came to imaging samples or processing images. I have learned so much from all of you. Louie Kerr and Drs. Carsten Wolff, Jim McIlvain, and Chris Bjornsson, you made me fall in love with microscopy even more and were always extremely helpful in anything as simple as getting microscope time or difficult as working through an imaging issue. Louie, especially, the work in this thesis would not have happened without your help. Marie Russell, you make the institute go round. Thank you for your tireless work in making sure our facilities ran, especially the insectary. Lastly, Sarah Mileikis and Briana Bertochi, I am immensely indebted to you both for helping coordinate and manage the day-to-day of the PhD. You both went above and beyond your duties to make sure everything ran smoothly.

Each summer, I had the privilege of being involved with the MBL research training courses. I have met, learned from, bonded with, and sometimes even taught, the Embryology and Physiology students as a graduate instructor. Drs. Carlos Patiño Descovich, Valentina Baena, and Davis Laundon, especially, you all were a massive help in several lab projects and a delight to work alongside. I am very thankful particularly to Drs. Carole LaBonne, Dave Sherwood, Nicole King, and Dan Fletcher for allowing our lab to have a place in these courses. In the Arthropod section, it was truly summer camp to uncover the fundamentals of oogenesis with Dr. Sally Horne-Badovinac and her team of co-instructors throughout the years, Drs. Seth Donoughe and Audrey Williams and Sierra Schwabach. Sally, you are one of my favorite people and it has been a joy to continue to work with you on the data we have collected these last three years and to consider you a close friend. Dr. Dave Matus, thank you for all the career and life advice around the break room table and your enthusiasm for any results I brought to you. Your love of science is a marvel and I am glad you have been able to be a role model for preserving that.

Especially important during the turbulent waters of the cross-country transition was the anchor of the Patel Lab. You all are great and I am so happy to see everyone succeed. Jessica Wilkins, I am so thankful for your help in Lepidoptera rearing and for looking after the *Plutella* colony in my stead. Dr. Ryan Null, I am forever in awe of the foundation you set in the scale development work, and I am glad to be able to contribute a small piece to cap your swallowtail story. Drs. Aaron Pomerantz, Rachel Thayer, and Dennis Sun, I wish we could have spent more time together in the lab, but I am amazed at what you have been able to accomplish and your growth as scientists and hope to see you more as we continue our careers. Aaron, I am glad to have still been able to tag-team the Lepidoptera side of the lab with you from different coasts and am so glad you have found the perfect career. Your work on glasswings was Herculean and your knowledge of sequencing and approach to research inspires me. Rachel, your work is always stunning and it has been great to have you as a role model and mentor. I hope I can pull you away from *Drosophila* work some time to collaborate on fun butterfly projects in the future. Dennis, you are a fountain of talent and I am so impressed by your ability to do whatever you set your mind to; thank you for your friendship these past years and looking forward to many more. Dr. Heather Bruce, thank you for always being down to have conversations about homology and morphological evolution that immediately veer into the weeds and gritty details. I am glad I can call you a friend and know your research is going to keep knocking it out of the park. Sophia Kelley, I feel so privileged that I was able to mentor and then quickly become your colleague in investigating all types of weird and wacky butterfly egg shapes. You will do great things in whatever you do!

Dr. Jenny McCarthy-Taylor, you get your own paragraph. You are such an amazing, diligent, and resilient researcher. It has been amazing to watch your progress in the lab as you started from just having an interest in developmental biology to becoming an absolute expert in any technique you set out to use. Your ability to troubleshoot and develop methods is such a gift. But, most of all, I am eternally grateful for your friendship and support after we moved to Massachusetts. Sharing this PhD process has been a joy and having you to lean on, a gift. I am so excited to see where

your future takes you and know that you will keep doing amazing science in the mollusk world. You have your vision and plan and you will accomplish it. Your star is bright!

Dr. Nipam Patel, thank you for giving me the freedom and having the trust in me to explore whatever avenues of research struck my fancy. Your advice and eye for detail throughout the dissertation process have doubtlessly improved the body of work I have been able to produce. I am grateful for your time and flexibility throughout the PhD and it has been amazing to see how you have advanced the mission statement of the MBL year after year, ensuring its position as a place to conduct cutting-edge science.

My research does not exist in a silo. It exists in its current state only through the collaborations I was lucky to build with colleagues from all spheres. To the butterfly jocks, Drs. Arnaud Martin, Joe Hanly, Luca Livraghi, Anyimilehidi Mazo-Vargas, and Owen McMillan and Ling Loh, and Remí Mauxion, thank you for the generosity in time, research advice, and use of facilities you have been able to give me as well as camaraderie as I worked on Lepidoptera in a marine lab. Dr. Paul Frandsen, it has been such a great experience to work with you and see some of the far reaches of the world together at the International Society for Trichoptera meetings. You made working with caddisflies feasible and fun; I am glad we get to work on *Nectopsyche* together. Dr. Ralph Holzenthal, I am indebted to your generosity, not just with specimens, but with a lifetime of advice when it comes to the *Nectopsyche* work. Likewise, Dr. Ernesto Rázuri-Gonzales thank you for all of your help throughout my learning phylogenetics. Dr. Bob Henricks, thank you for showing me the ropes and getting your boots wet with me as I learned to collect Virginia caddis. Dr. Alison Tebo, thank you for your generosity in hosting me at your lab at Janelia. It was like being a kid in a candy store and I hope we will get to work together again soon. Fred Gagnon, your knowledge and dedication to butterfly rearing astounds me and the research in the second chapter would not have happened without your impeccable stewardship of Magic Wings. Likewise, Rich Cowen, thank you for the accommodations in the weekly pupae shipments and your help in tracking down suppliers.

Jeremiah, you are my best friend and I am lucky to have you in my life. Your support and patience throughout the PhD cannot be understated. You inspire me to try my hardest and be true to myself and I can only hope to follow your example.

Introduction

> *The shape and figure also of scales are very various—some being long and slender; others short and broad; some nearly round; others oval, ovate, or oblong; other spathulate; others panduriform or parabolical; some again almost square or rhomboidal; many triangular; some representing an isosceles triangle, and others an equilateral one; lastly, some are lanceolate and others linear; again, some have a very short pedicle and others a very long one: with regard to their extremity; some are intire[sic], without projecting points or incisions, while others are furnished with them: of these some terminate in a single long mucro, others have several shorter ones; some are armed with teeth, varying in number from two to thirteen in different species. Many other forms might be enumerated, but these are sufficient to give you a general notion of the infinite variety of this part of the works of the* CREATOR. (p. 647)
>
> -William Kirby, M.A. F.R. and L.S.
>
> from Kirby & Spence 1826, *An introduction to entomology: or, Elements of the natural history of insects, Vol. III*

It is a truth universally acknowledged that a biologist, in search of a way to establish their subject's importance, must be in want of a quote invoking a creator to explain the extent of its diversity. And so, as in the quips of JBS Haldane, this dissertation embarks with a description by William Kirby of the multitude of "shapes and figures" butterfly and moth scales assume. Meant to impress the daunting task taken on when attempting to grok the variety of the natural world, this line likewise highlights how far back an interest in the vestiture of arthropods stretches. Antoni van Leeuwenhoek himself remarked on the "feathers" of the silk moth, stating that "[he does] not remember that [he's] seen them of so curious a make in any other flying insect" (Leeuwenhoek 1800). Following from this passion at the onset of our ability to observe this curious dandruff in any appreciable detail, there has since been significant effort to characterize insect plumage.

Categorization in light of infinite variation

As can already be gleaned, a lion's share of the correspondence given to these cuticular projections has concerned the Lepidoptera, in no small part due to the function their scales serve in producing coloration and pattern on the wings and body. The first researchers of butterfly color sought a perfect blue pigment as they ground iridescent wings with mortars and pestles, just to be met with brown powder; and only through careful attention by comparison under water and air, were they able to link differential surface refraction to their brilliant wavelengths—some of the first description of non-pigment derived, or structural, color (Hooke *et al*. 1665). Since, scales have been a wellspring of both optical analysis and inspiration for the physics-minded, dissected to understand the manner they split light in relation to an insect's and, with some hope, our, agency (Ghiradella 1984; Greanya 2015; Biró & Vigneron 2010; Dolan *et al*. 2014). These

studies do well as examples emphasizing the extremely tight connection scales have between their structure and their function, with nanometer differences able to produce pronounced shifts in color (Thayer *et al*. 2020).

In an impressive treatise aiming to coordinate description, Downey and Allyn assemble a nomenclature for butterfly and moth wing scales which importantly delineates structure as a collection of characters and their corresponding states, providing a lexicon for traversing morphological differences (1975). This work heavily draws from the body of taxonomic description capitalizing on the variety in scale form to a systematic end. In equal parts for deep evolutionary splits and finer branches between nascent diverging populations, differences in scale type have demonstrated themselves as markers of unique lineage (Zhang *et al*. 2018; Lavitt 2016). Similarly, in ants and mosquitos, nomenclatures have been crafted to take advantage of the taxonomic utility in exoskeletal vestiture (Bolton 1994; Harbach & Knight 1980). Yet, these systems remain mutually unintelligible and, at times, contradictory. Systematists attempting to combine large cross-ordinal level datasets of morphological characters, therefore, find themselves facing a tower of Babel.

A solution brought forward by the taxonomic community has been to consolidate these patois into an ontological network, therein systematically categorizing relationships between subject areas. In perhaps the first major cross-taxa effort, Girón *et al*. reconcile descriptive terms for the cuticular skeleto-muscular anatomy across Insecta into tiered, branching concept trees that allow the direct comparison of far-separated lineages in common language (2023). As an ideal, a biological ontology should link concepts that directly reflect either homologous or mechanistic relationships, and while this resource represents a significant leap in crosslinking disciplines by establishing a common ground, in its current state its utility rests in its ability to provide a scaffold for generating evolutionary questions instead of reflecting them itself.

As an example, in the current network, appendages, spurs, and setae are linked as either multicellular or unicellular cuticular evaginations united under the umbrella of cuticular protrusions. In a separate branch, cuticular invaginations are given the subcategories of apophysis, cuticular tendon, and branching cuticular invagination. In such a scheme, there would be no way for a parsimonious relationship to be established while maintaining groupings by homology when a structure or its descendent transforms as a result of a transition in mechanism from an invagination to an evagination. This is problematic, especially when such developmental transitions have proven to be immensely important for morphological innovations and the understanding of homology relationships, for example, in the evolution of mammalian hairs and glands from early-branching vertebrate scales (Oster & Alberch 1981). Furthermore, linking multicellular and unicellular evagination muddies the extent to which any branch can be illustrative of mechanism when these processes are undertaken through the deployment of very different cellular programs (Pastor-Pareja *et al*. 2004; Aldaz *et al*. 2013; Keeling *et al*. 2016; Burgoyne *et al*. 2015).

Whereas this ontology scheme captures the spirit of morphological homology, it is subject to the biases of the data used to build it, namely taxonomic descriptions of structures that historically

have neglected the developmental basis of traits in question. If the goal is to determine how observable variation in form is realized, and to infer patterns of evolution between particular shapes, scales and other epithelial projections must be considered wholly as biological units influenced by ontogenetic processes.

Scales in their evolutionary-developmental context

Scales are a type of 'macrochaete', a developmental subclass of structures defined as the secreted product of an apical extension derived wholly and uniquely from a single epithelial cell. All arthropod macrochaetae arise from the same 'Sensory Organ Precursor' cell lineage, whereby a mother SOP cell is derived from an epithelial precursor and then differentiated throughout one or several divisions into external (macrochaete, 'trichogen'; socket, 'tormogen') and internal (neuron; sheath, 'thecogen') components of sensory organs, broadly defined (Hartenstein & Posakony 1989). Some sensory organs neglect to form their neural component, through a shift in the pattern of their cell division, apoptosis of a daughter cell, or otherwise, and thus possess no sensory function despite their name and exist only as the macrochaete held in pitch by its accompanying socket cell (Lai & Orgogozo 2004). As an example, most lepidopteran wing scales fall into this "non-innervated" category (Clever 1958). If a sensory organ retreats into the body wall instead of producing an extension apically, it forms an internal sensory structure; despite their shared ancestry, these structures are not considered as macrochaetae and instead fall into the category of 'chordotonal organs', the most famous of which being Johnston's organ, a mechanoreceptive sensory organ which serves essential roles in receiving sonic and vibrational stimuli to the insect antenna (Eberl *et al*. 2000).

Macrochaete morphogenesis and genetic specification is best known through study of *Drosophila melanogaster* bristles, particularly those of the dorsal thorax and wing margins. In these tissues, initially segment polarity and gap gene-specified proneural clusters begin to express members of the *achaete-scute* complex of transcription factors (Parks *et al*. 1997). Within these subsets of cells, future SOPs specify through a Delta-Notch mediated lateral inhibition process whereupon enrichment in Delta reinforces *achaete-scute* transcription factor expression within the destined SOP while Notch signaling in neighboring to-be epithelial cells activates a transcriptional cascade involving *Suppressor of Hairless* and *Enhancer of split* that downregulate achaete-scute activation (Culí *et al*. 2001). This feedback loop eventually manifests a strongly Delta-expressing SOP defined by *achaete-scute* expression surrounded by a Notch-positive epithelium. In olfactory sensilla, as an example, *achaete-scute* expression subsequently activates downstream transcriptional networks including *cato* and *asense*, genes of particular interest since chordotonal organs—specified upstream by proneural *atonal* expression in place of *achaete-scute*—also enlist these factors, emphasizing their importance in establishing neural, support, and glial cell fates and the repetitive use of genetic cassettes in these signaling networks to produce similar morphological outcomes (Vervoort & Ledent 2001).

Activation of these downstream factors results from tightly coordinated spatiotemporal expression and inhibition of Notch by daughters of the SOP facilitated by asymmetric cell

division. The initial division of the SOP results in daughter cells designated pIIa and pIIb, with the former giving rise to the tormogen and trichogen and the latter dividing into the thecogen and neuron (Hartenstein & Posakony 1989). pIIb asymmetrically inherits Numb, an inhibitor of Notch, leading to comparatively lower expression of this factor within this cell; in this manner, total knockdown of Notch expression results in sensory organs entirely composed of neurons (Rhyu *et al*. 1994). Likewise, as pIIa divides, the trichogen asymmetrically inherits Numb and Numb^{-} mutants produce four tormogen cells in a cluster (Couturier *et al*. 2012). The orientations of the mitotic spindles in these asymmetric divisions are important to these outcomes and are understood to differ between the SOP division, where it seems influenced by Pins-Mud signals in the anterior Numb-expressing pole and Frizzled-Daschous and Bazooka-Par6-aPKC in the posterior, and the pIIb cell, where *inscuteable* seems to play a more central role under possible persisting influence from Frizzled (Schweisguth 2015; Roegiers *et al.* 2001). While the orientations of these divisions seem to be well-conserved amongst insects and suggest that their molecular underpinnings may be widely shared, genetic dissection using RNA interference in the *Tribolium castaneum* system has highlighted the degree to which upstream transcriptional networks can dramatically differ from the combinatorial manner sensory organ identity is laid out in *Drosophila* proneural clusters (Lawrence 1966; Gho *et al*. 1999; Klann *et al*. 2021). Overall, whereas our deep understanding of sensory organ development comes from intensive analysis within *Drosophila*, a common theme arises where assumptions mislead investigations if this genus is taken as the only reference; therefore, a modern study design now uses *Drosophila* as a 'hub' with multiple traverses to-and-from it and species of interest to assess the degree of universality for a given mechanism or pathway.

The genetic control and cytoskeletal organization of *Drosophila* trichogen development post-pIIa division, in particular, is reasonably well understood and has created a fertile ground for extending a comparative lens to development of macrochaetae in other species of insect (Tilney & DeRosier 2005). The tilted axis of pIIa division positions the trichogen below the epithelial layer underneath the tormogen where it begins endoreplicating its nuclear DNA between two to three times, producing copy numbers between 8 and 16C (Audibert *et al*. 2005; Hanson *et al*. 2005). It is not known whether this polyploidization involves the entire genome or only a portion, but it is assumed that this replication is important for supplying raw materials for growth as deficits in nuclear copy number, produced by knocking out E2F and Rbf, align to smaller, stunted macrochaetae (Weng *et al*. 2003). Subsequent growth is rapid, with an apical projection erupting from the trichogen and resting against the socket cell which nearly but not entirely encircles the elongating shaft (Lees & Waddington 1942).

This primary extension is decorated with microvilli along its leading front which contain a crosslinked actin filament core that runs from the plasma membrane into the cytoplasm and is subsequently stabilized into cortical bundles by the activity of forked—it follows that differential expression of forked between the trichogen and tormogen ensures that only microvilli in the former will stabilize and grow out to form bundles (Tilney *et al*. 2004). Cortical bundles extend through actin polymerization and in doing so propel growth at the distal tip of the trichogen (Tilney *et al*. 2000). Within the shaft of the developing bristle, away from the elongating tip,

initial forked-formed bundles themselves undergo additional crosslinking into modular subunits bound by fascin, encoded by the gene *singed* (Tilney *et al*. 1998). These individual modules subsequently fuse first as a property of overlap and cross-linking but are further smoothened *via* actin polymerization within the remaining gaps between segments, primarily fused by forked proteins (Guild *et al*. 2003). The resulting cortical bundles are cross-linked, hexagonally packed arrays and incredibly long, up to 400μm at the peak of bristle elongation, and circumferentially attached to the plasma membrane in groups of seven to eleven, depending on the bristle diameter (Tilney *et al*. 1996). Although initially documented as only occurring from the tip, observations first in the arista—a macrochaete similar to a bristle but with branches along its shaft—but later replicated in thoracic bristles, determined actin treadmilling in individual bundle modules contributes to overall length (Fei *et al*. 2002). As these units are themselves able to increase in size, albeit slowly, the full extent of the projection elongates akin to pulled taffy, parsimoniously explaining how the tapered point becomes more severe as development climaxes. Once the maximum extent of the trichogen has been reached, the cortical bundles are no longer maintained and degrade through a process of actin turnover as the cell secretes the cuticular husk that will remain in the adult imago once the trichogen has completely retracted and delaminated from the epithelium (Guild *et al*. 2002).

Secretion of the cuticle is thought to be mediated by microtubule trafficking networks that cargo, among other factors, the Rab11-localized Dusky-like which permits stable deposition of cuticle through plasma membrane-chitin binding (Nagaraj & Adler 2012). Despite their importance at the end stages of bristle development, treatment with the microtubule depolymerization drugs nocodazole, vinblastine, and colchicine and the microtubule stabilizer taxol do not have any effect on elongating bristles (Tilney *et al*. 2000). Only when microtubule antagonists like vinblastine are introduced prior to bristle budding do deficiencies in biased axial growth become apparent, in which case bristles grow shorter and stout (Turner & Adler 1998). In fact, closer analysis of the initial apical projection of the trichogen reveals primary reinforcement by the microtubule cytoskeleton before formation of the microvillus (Keil 1997). Altogether, these experimental results, along with the observation that the microtubules in the bristle are all relatively short, imply that the bristle microtubule cytoskeleton is established early on and exists as a stable population of mixed orientation with little to no rearrangement, elongation, or turnover within the shaft; however, the total microtubule population may be important in establishing cytoplasmic bulk essential to developing proper morphology, and comparison can be made between areas of varying width in the elongating bristle shaft and microtubule density that seem to corroborate this link (Tilney *et al*. 2000). Genetic dissection has refined the role of microtubules and uncovered Ik2 and Spn-F as necessary factors in trafficking cargo to the elongating tip, with defects in these proteins producing phenotypes where gene products are not properly localized along the length of the shaft (Bitan *et al*. 2010). Therefore, in addition to the mixed-orientation microtubule population that contributes to cytoplasmic bulk, there coexists a subpopulation of microtubules with distally oriented minus ends that determines a bristle proximo-distal axis, explaining the previously contradictory axial patterning defects produced from pharmacological manipulations.

In part due to this obvious effect on appearance, along with pigment genes, factors influencing bristle morphology were some of the first to be characterized and harnessed for their use as markers in genetic crosses (Lees & Picken 1945; Tinderholt 1960). Since, a litany of genes have been identified in both gain- and loss-of-function screens with effects on bristle number, distribution, polarization, and morphology, among other traits (Abdelilah-Seyfried *et al*. 2000; Mummery-Widmer *et al*. 2009). Included in the gene products of the identified candidates are factors constituting an assortment of actin and microtubule binding proteins, along with a variety of crosslinking factors that mediate forces between these cytoskeletal polymers. Actin binding proteins in particular are known to have dramatic influences on the rate of polymerization of microfilaments, their branching, and stabilization, but they can also influence the stiffness of cortical bundles, impressing the need to think of the aforementioned dynamics described for the elongation process with an eye to physical interactions that may also contribute to the final structure (Pollard 2016; Claessens *et al*. 2006). In fact, using this interdisciplinary approach, the curved morphology of thoracic bristles can be explained by considering physical interaction with the overlying cuticle as individual modules of the cortical actin bundles are affixed together with forked protein—"locking in" this bend in place along the growing shaft (Tilney *et al*. 2004).

Whereas much less is known about the genetic control of bristle morphology outside of the *Drosophila* clade, descriptive studies focused primarily on cytoskeletal organization have highlighted macrochaete development in a greater diversity of insects, particularly culicid and lepidopteran scales. Mosquitoes are a convenient group to ask questions regarding the differences underlying bristle and scale morphologies because they share a close evolutionary relationship with *Drosophila* as dipterans, have significant genetic resources invested to their study due to their links to human health and diseases, and sport a mix of scales and bristles on their thorax. Opposite to what may be expected from their relatively recent shared ancestry, actin polymerization seems to not be required for elongation in budding mosquito scales, as destruction of microfilaments with cytochalasin D at early stages of growth produce dramatically shortened bristles, as is seen in *Drosophila*, but longer, narrower scales (Djokic *et al*. 2020). This effect solely on scale width is recapitulated in *forked* knockouts in *Aedes aegypti* which share a similar phenotype to the cytochalasin D treatment. It seems, then, that actin dynamics in yellow fever mosquito bristles follow *Drosophila* paradigms, but fundamental differences have taken place in the differentiation of scale morphologies within this species. Similarly, factors associated with the microtubule cytoskeleton in *Drosophila* seem to have divergent and specialized functions in *A. aegypti*. Spn-F, as aforementioned, has been shown to be important in localizing cargo to the tip of developing *Drosophila* bristles, as well as demonstrating a possible role in mediating interactions between microtubule minus ends and cortical actin networks (Abdu 2006). Knockout of Spn-F in *A. aegypti* has no effect on typical spatulate scales, but a distinctive bulbous or skewed tip phenotype appears in bristles and specialized falcate scales that have a tapered apex (Djokic 2021). This defect is linked wholly to clumped aggregations of microtubules along the distal tips of the bristles and falcate scales, with no differences in actin organization detectable. Again, specialized mosquito macrochaete morphologies present altered interactions between cytoskeleton-interacting partners.

Oddly, despite being more distantly related to *Drosophila* than mosquitoes, lepidopteran scales do require actin polymerization for elongation since treatment with cytochalasin D completely prevents any significant scale outgrowth when introduced when scales start to bud (Dinwiddie *et al*. 2014). Furthermore, cortical actin bundles localized to the abwing surface prefigure where ridges will form; specifically, in the spaces between where this bundle population attaches to the plasma membrane. Later in development, in these spaces between and adjacent to these nascent ridges, cortical actin composed of ultrafine filaments spatiotemporally correlates to microfeatures of the imaginal scale ultrastructure including microribs and nanotips along and atop ridges, respectively, and crossribs surrounding areas of thinner cuticle deposition that will perforate upon eclosion to reveal windows into the scale interior (Lloyd *et al*. 2023 preprint). Even papilionid scales with honeycomb-like nanostructural arrays between adjacent ridges instead of consecutive rectangular windows follow this seemingly hierarchical shuffling of fine microfilaments in late-stage scale development, pointing to a common mechanism, namely cortical actin reorganization, that may be important in diversification of scale ultrastructure between butterfly families (Seah & Saranathan 2023). Snapshots into the development of other specialized scales, especially those responsible for vibrant structural coloration, implicate other cellular structures and organelles apart from the cytoskeleton in nanostructure fabrication, too. For example, plasma membrane buckling has been hypothesized to drive the creation of precisely spaced ridges along the lamellae of ultraviolet-reflecting *Colias eurytheme* scales and the convolution and subsequent deposition of chitin along an endoplasmic reticular scaffold seems to produce green-reflecting gyroid crystals within the lumen of scales on the ventral hindwing of *Callophrys* hairstreaks (Ghiradella 1974; Ghiradella 1989).

The specific dynamics of how the actin cytoskeleton organizes in Lepidoptera with comparison to *Drosophila* bristles is still in need of further study that reaches beyond correlations gleaned from serially fixed tissues; albeit, from these detailed morphological studies, many features seem to hold constant between the two clades including the phases of actin bundling, ridge formation, and association between ploidy level and macrochaete size (Picken 1949; Stossberg 1938; Cho & Nijhout 2013). The most obvious contrast between the development of *Drosophila* bristles and lepidopteran scales is in the flattening process which occurs shortly after the scale bud reaches a critical length and which has been suggested to correspond with a redistribution of the microtubules to the cellular surface from a previously longitudinal orientation, but the link between this shift in organization and overall scale morphology remains unclear (Overton 1966). Interestingly, flattened mosquito and lepidopteran scales both share an asymmetric distribution of cortical bundles around their perimeter, with larger bundles localized to the smooth lower lamina of the scale and smaller bundles adhered to the ridged upper lamina (Djokic 2020; Ghiradella 2010). Cylindrical *Drosophila* bristles, by contrast, have reduced to absent cortical bundles in the superior quadrant only, with equally sized, robust bundles in the remainder of the circumference (Tilney *et al*. 2004). The identical organization of actin cortical bundles between the similarly shaped scales of the Lepidoptera and Culicidae indicates how shared motifs of cellular design can arise from divergent interactions of its constituent parts.

The first of two crucial points to reiterate regarding macrochaete developmental heritage is that, regardless of their relatively massive size, all are the remnants of one cell, exemplifying a "one cell-one structure" principle that differentiates them from similar-appearing, non-macrochaete structures that form from cellular apical extensions. For instance, a single cell is capable of producing multiple wing hairs which erroneously appear to be discrete, independent structures when viewed in the adult (Richards & Richards 1979; Guild *et al*. 2005; Lu *et al*. 2015; Whitten 1973). The second point is that morphologically diverse structures such as bristles, setae, chaetae, and the like are all macrochaetae, and so, by fact of belonging to this cell lineage, these single cell-derived epithelial extensions in turn share ancestry to scales (Galant *et al*. 1998; Zhou *et al*. 2009). Defining a scale as a lamelliform macrochaete and looking through an evolutionary lens, scales have arisen many times within arthropods, with examples present in the arachnids, diplopods, Isopoda, Collembola, Zygentoma, Archaeognatha, Orthoptera, Psocodea, Coleoptera, Diptera, Trichoptera, Lepidoptera, and very probably other orders, as well (McCoy *et al*. 2019; Eisner *et al*. 1996; Verhoeff 1901; Zhang *et al*. 2014; Larink 1976; Yang & Yen 2001; Enderlein 1906; Beal 1998; Westphal-Ferreira & Navarro-Silva 2017; Robertson & Holzenthal 2008; Kristensen 1970). These instances are parsimoniously considered to be independent acquisitions of scale-like characteristics and the ancestral state believed to more closely resemble a bristle, that is, a mostly cylindrical macrochaete with no apparent flattening.

Approach of this thesis

This dissertation tackles just one small piece of the macrochaete evolution imbroglio, choosing to focus on the superorder with the most well-characterized scales, the Amphiesmenoptera. Its first chapter aspires to replicate the excitement of the first microscope-aided glimpses of scale structures by developing methods to capture high resolution, real-time images of cellular activity within growing macrochaetae from living pupae. Its second chapter hopes to contribute to our understanding of one axis of scale shape heterogeneity by identifying cytoskeletal and genetic correlations in cylindrical macrochaete morphologies throughout the lepidopteran lineage. Finally, its third chapter develops tools and sets the stage for future analysis of a remarkable genus of caddisfly which has evolved scales and elaborate color patterns reminiscent of butterflies and moths and places these convergent traits into a phylogenetic context.

Chapter I: Transgenic-free *In Vivo* Visualization of Cytoskeletal Dynamics and Organellar Structure in Lepidopteran Scales

I.1 Introduction

Butterfly and moth wing patterns are mosaics composed of hundreds of thousands of scales, which are the secreted chitinous remnants of single extremely large and polyploid epithelial cells which develop in the pupa (Nijhout 1991). Despite generally following a blueprint of an upper (abwing) and lower (adwing) lamina separated by a lumen—although early-branching moths lack this open cavity and are instead completely solid—lepidopteran scales have evolutionarily radiated with respect to these features in shape, integrated pigments, and accompanying micro- and nanostructural elements (Kristensen 1970; Ghiradella 1985). Still, since they are unicellular in origin, the morphology of adult scales directly correlates to a terminal differentiated shape of an individual pupal trichogen cell. This linkage between cellular development and form in the evolutionarily and structurally diverse lepidopteran scales provides a unique opportunity to link morphogenesis to physiology at the level of single cells.

Due to their usefulness as taxonomic characters and certain structural photonic properties exhibited in derived scales to produce iridescent colors, a wellspring of interest in scale morphological description has outpoured from the taxonomic and physical sciences spheres, but this has not been mirrored in investigations of scale development or growth (Downey & Allyn 1975; Barrows & Bartl 2014). This is in part due to the diligent labor required for serial dissections, which still only capture snapshots of cellular organization and can struggle to document transitions between developmental time points of frozen dynamic states (Overton 1966). Still, from these investigations, it has become clear that the organization of the actin cytoskeleton in particular serves as a template for such scale features as the abwing ridges, microribs, and lamellae, and that evolution acts on the cortical actin network to produce derived features in diverse, structurally colored, scale types, such as honeycomb lattices in ultrablack swallowtail scales (Dinwiddie *et al*. 2014; Lloyd *et al*. 2023 preprint; Seah & Saranathan 2023). An important takeaway from these more recent studies, too, is that the actin cytoskeleton late in development is highly dynamic.

The development of live imaging techniques has served to contextualize biological processes and provide insights beyond what would be possible with fixed tissues approaches. As an example, the discovery of vesicle trafficking along microtubules, and subsequently the motor protein kinesin, was enabled by innovations in live DIC imaging of axons and protists (Allen *et al*. 1981; Vale *et al*. 1985). Similarly, live imaging of cells dividing to become bristles in *Drosophila* nota, themselves homologues of lepidopteran scales, have permitted investigation and perturbation of asymmetrically distributed factors specifying trichogen vs neural fates (Galant *et al*. 1998; Zitserman & Roegiers 2011; Roegiers *et al*. 2001). Unlike in *Drosophila*, however, live imaging has been impeded in Lepidoptera by the presence of a usually opaque pupal cuticle and the difficulty of keeping transgenic lines due to a predisposition towards inbreeding issues within colonies (Saccheri *et al*. 1996). Additionally, transgenic approaches would prove laborious in

cases where it is of interest to assess the diversity of structures throughout a range of species, since each new species added would require the concomitant development of specific genomic resources and gene editing protocols. These issues were circumvented by the discovery of manipulations performed right after pupation occur that take advantage of the softened pupal cuticle to move the not-yet-fused wings apart to reveal the ventral forewing and dorsal hindwing surface (Iwata *et al*. 2014). The addition of commercial live dyes into the wing at this state allows the staining of cellular components in the early developing wing and resolution of some subcellular features without relying on transgenically expressed proteins, too—a boon for comparative studies between species (Ohno & Otaki 2015). Further modification of this technique entailing injection of dyes and labeled proteins into the hemocoel of the thorax expanded the range of labeling options and reached later stages of development than soaking (Nakazato & Otaki 2023). Along a different axis, speckle-correlation reflection phase microscopy was applied to developing butterfly scales to resolve microfeatures at high resolution (McDougal *et al*. 2021). This form of imaging produces optical contrast by differences in refraction indices across adjacent interfaces of cellular components, meaning that development can be resolved in a label-free manner (Choi *et al*. 2014). These approaches do not come without drawbacks, however. The wing soaking and body injection use large volumes of high concentration dyes to achieve staining and are not optimized for high resolution microscopy or long imaging periods. Furthermore, this technique necessitates raising species as caterpillars since specimens must be processed immediately following pupation, which can be impossible in cases where a host plant is unavailable or rearing space is limited. In a similar vein, speckle-correlation reflection phase microscopy avoids constraints of dyes and achieves high contrast but suffers from the double-edged nature of label-free imaging in that it is not possible without secondary validation experiments to precisely identify structures.

This paper describes a suite of mounting protocols, staining approaches, and imaging parameters that allow the continuous imaging of lepidopteran scales throughout the extent of their morphogenesis. By mounting wings flat, objectives with higher resolution and optical magnification can reach areas of the hindwing and direct injection of live dyes both reduces total volume used and produces brighter signal since the stain is introduced at the relevant developmental timepoint instead of having to persist from an early dye loading event conducted in the fresh pupal wing. Dyes have been tested that label the nucleus, lysosomes/acidic vesicles, mitochondria, cell membrane, and actin and microtubule cytoskeletons, which can be used in combination or separately to continuously resolve sub-cellular localization and organelle interactions throughout morphogenesis. Imaging conditions for confocal, two-photon, and STED microscopy are outlined to allow breadth in fluorophore unmixing, laser exposure, and targeted resolution. Together, these protocols are used to explore the cellular lineage of sensory organ precursors that give rise to scale cells in two species of moth and a butterfly, to outline the growth phases in early scale elongation while resolving in particular actin filament mechanics, and uncovering late-stage actin remodeling dynamics in structurally colored swallowtail scales that display a strikingly derived actin cytoskeleton.

As a soapbox, the data collected in this chapter primarily capitalizes on the *Plutella xylostella* (diamondback moth) system which I have brought into the lab and utilized as a developmental workhorse. To proselytize, *P. xylostella* can be reared in large numbers while maintaining high fecundity on completely artificial diet and has the shortest generation time of any non-mining lepidopteran, able to develop from egg to adult in as little as 11 days (Liu *et al*. 2002; Danks 2012). Importantly for this study, it also possesses an optically transparent cuticle overlying its wings that, aided with refraction index-matching Halocarbon oil, allows complete visualization of wing morphogenesis from the onset of pupation (Fig.I.1). That being said, it should be reiterated that the protocols herein were intentionally developed to be species-agnostic and I include data from three other lepidopteran species to illustrate this point.

I.2 Methods

I.2.1 Animal husbandry

Plutella xylostella strain G88 ('Geneva 88 – susceptible') was generously gifted from Anthony Shelton (Cornell University) in January 2018 and has since been maintained at either 25°C or 27°C under a natural or 16:8 (day:night) light cycle, respectively. Larvae are fed exclusively on diamondback moth diet with supplemented linseed oil (Southland Products Incorporated) in Styrofoam cups whereas adults feed on a 12% sucrose solution dyed with yellow food coloring. *Trichoplusia ni* were ordered from Frontier Agricultural Sciences (L9282) and reared on the supplied diet until pupation. *Junonia coenia* were initially purchased from Shady Oak Butterfly Farm (Brooker, FL, out of business) and raised on locally collected *Plantago lanceolata*. Later, a lab-adapted colony was gifted by Fred Nijhout (Duke University) and maintained on multiple species diet (Southland Products Incorporated) mixed with powder made from dried *P. lanceolata* leaves. *Papilio palinurus* pupae were reared at various butterfly farms in the Philippines and purchased weekly through London Pupal Supply, LLC under USDA Permit P526P-22-07598 and housed in approved facility 5046.

I.2.2 Mounting, modified wing flip, and fenestration protocols

To prepare diamondback moth pupae for injection, a strip of double-sided sticky tape was adhered along the length of a microscope slide to which appropriately aged *P. xylostella* pupae were aligned with their left wing facing up. Two spacers made of 5-6 Corning 24mmx24mm #1, or equivalent height, coverslips adhered together with nail polish flanked the line of pupae and stuck to the tape such that their outer edge was flush with that of the microscope slide. An additional square of double-sided sticky tape was attached to the top of each spacer before injection began.

The method described by Iwata *et al*. permits the visualization of developing ventral forewings as a flat surface, but the hindwing remains curved around the pupal body, making it difficult to access certain areas of the wing with higher magnification/higher numerical aperture objectives with short working distances—impeding the investigation of all scale types across the wing at high resolution (2014). Instead, for this study, to investigate hindwings of non-transparent pupae, a new method was developed where caterpillars were processed immediately upon their ultimate

molt before the pupal wings had fully expanded: flipping both the hindwing and forewing together onto a square piece of Parafilm through a rectangular slot cut ~3/4ths of the length up and reaching ~3/4ths of the width. The forewing was then carefully pulled off the now completely flat hindwing adhered to the Parafilm surface and tucked under the skinny arm of the slot to prevent it from flipping back onto the hindwing. ~5µL of 400µM phenylthiourea in Grace's insect media (Gibco) with 10x supplemented antibiotic/antimycotic (Gibco) was dispensed onto the exposed hindwing surface to prevent melanization of the hemolymph and a square of ethanol-sterilized Saran wrap pressed atop to seal in moisture (Sup.I.1). The pupa with its hindwing sandwiched between the Parafilm and Saran wrap was then flipped onto the lid of a 27mm Nunc glass bottom dish and placed into a Tupperware lined with paper towel wet with distilled water and a dab of soap until ~3 hrs before the desired time point for injection (the Tupperware filled with pupae can be placed in an incubator to speed development, or chilled to slow growth, in which case the soap in the paper towel prevents the growth of mold). At 25ºC, scales were found to begin to erupt around 72hrs after pupation in *J. coenia*. This novel mounting allows equal access to all areas of the wing as close as the natural limit of the peripodial membrane allows since the entire hindwing is now pressed against the glass surface. Further modifications wherein both wings are flipped onto a piece of Saran wrap instead of a piece of Parafilm to image the ventral surface of the hindwing are possible, too.

For species that cannot be reared in the lab due to lack of host plants but can be purchased as pupae through butterfly house suppliers, or for pupae where the critical period right after pupation has been missed, a live dissection of the forewing is possible to still allow imaging of hindwing development (Sup.I.8). To prepare 'fenestrated' pupae, first VALAP (1:1:1 vasoline, lanolin, paraffin wax) is melted in a Falcon tube suspended in boiling water and used to affix the chrysalis to a microscope slide. A sharp, ethanol-sterilized scalpel is then used to carefully remove the forewing cuticle, making sure not to nick the developing legs or antennae along the pupal midline. If these structures are damaged or severed, it may be possible to get objectives closer to the wing surface, precluding the need for high working distance lenses, but wing development will be unpredictably delayed to accommodate wound healing processes which can make staging or the capturing of ephemeral events difficult. Removing the forewing cuticle with forceps, a twisted piece of Kimwipe is used to soak onto the gelatinous developing forewing and pull it up and back over the head of the pupae, at which point its hinge can be severed using the scalpel or a pair of eyelash microscissors. Quickly, pooled hemolymph from the forewing is sopped up using a clean KimWipe and the exposed pupal cavity washed thoroughly with ~175µL—for large swallowtail pupae—of 400µM phenylthiourea in Grace's media supplemented with 10X antibiotic/antimycotic to prevent melanization. This solution should be pipetted up and down within the pupal opening to completely wash and distribute the phenylthiourea solution and maintain the transparency of the hindwing peripodial membrane. Immediately after washing has finished, the fenestrated pupa should be injected, minimizing oxygen exposure and reducing bleeding time.

I.2.3 Staining and injections

Injection mixes were composed of a ratio of 12 parts anhydrous dimethyl sulfoxide (DMSO) to 3 parts unsupplemented Grace's insect media (Gibco) to 1 part stain at its respective stock concentration. If a stain recommends co-introduction with verapamil to discourage cellular exocytosis, 1 part 10mM verapamil replaces 1 part of the DMSO; likewise, if double or triple staining is desired, these parts are subtracted from the DMSO factor such that each part is equal to one-sixteenth of the total volume. Stains used in this investigation include SiR-Actin/SiR-Tubulin (Spirochrome), MitoTracker Red (Invitrogen), and LysoTracker Red (Invitrogen) at 1mM stock, and Hoechst33342 (Cell Signaling) at 1µg/mL stock to respectively stain the actin/microtubule cytoskeletons, mitochondria, acidic vesicles, and nuclei. SiR-Actin/SiR-Tubulin injection mixes were supplemented with verapamil, as recommended by the supplier.

Injections were completed using a filamented 1.0/0.58 (OD/ID) borosilicate needle pulled using the 'ZIKA Research and Mosquito Egg Injection' program from the Sutter Instruments 2018 Pipette Cookbook (Oesterle 2018). Prior to injecting the dye mixture, diamondback moth pupae must be paralyzed to prevent them forcing the liquid out of their pupal cavity. Unlike in vertebrates, paralysis by tetrodotoxin is not lethal and has no effect on insect metamorphosis, merely preventing muscle movements (Williams 1968). As such, in addition to preventing expulsion of injected liquids, fugu toxin has the added benefit of preventing twitching during image acquisition—making it possible to accurately resolve subcellular structures. 2.0µL of 1mM tetrodotoxin citrate (Abcam, ab120057 discontinued) dissolved in distilled water is dispensed into the needle using a 20µL capacity epTip and shaken vigorously to remove any air bubbles after which the tip of the needle was broken to a sharp edge approximately 0.5mm from the tip using fine Dumont #5 forceps. A Narishige IM-300 microinjector was attached to the capillary needle using the stylus pen and gasket and set to 13psi at a duration of 20msec. *P. xylostella* pupae were injected with the paralytic on their dorsal side underneath the tergite of abodminal segments III or IV. Within five minutes, the moth pupae should no longer respond to abdominal prodding with a pair of forceps. At this point, a new glass capillary needle was re-loaded as before with 0.8-1.8µL of the injection mix and administered to the medial posterior margin of the left forewing and at the posterior side of the distal wing tip. Wing flipped pupae do not require paralysis before dye injection and thus were carefully flipped off the dish lid and cradled in a horseshoe-shaped piece of clay before removing the Saran wrap covering the hindwing. Even more simply, fenestrated pupae do not require any additional processing after washing the hindwing surface and before injection and can be injected as-is affixed on the slide. Wing flipped and fenestrated pupae were injected in a quincunx pattern through the overlying peripodial membrane, centered around the region of interest for imaging. For all three types of mounted pupae, each injection consisted of depressing the foot pedal a maximum of three times per site until a spot of distorted liquid was visible inside the pupal cuticle that subsequently incorporated into the wing hemolymph.

After injection of *P. xylostella* pupae, they were left to rest for thirty minutes before a 24mmx60mm #1.5 coverslip was uniformly coated in its medial 50% with Halocarbon 700 oil and carefully placed onto the spacers, making sure to adhere it firmly to the double-sided tape

atop each. Then, with a pair of closed, blunt forceps, the glass above each pupa is stroked to ensure even coverage of the oil and optimal transparency. Following injection of wing flipped pupae, a drop of 400μM phenylthiourea in Grace's insect media supplemented with 10X antibiotic/antimycotic (Gibco) is placed onto the exposed wing surface of interest and the entire pupa very carefully and gently flipped onto a #1.5 Nunc 27mm glass bottom dish. Clean cotton saturated with distilled water is then padded around the perimeter of the dish with care not to touch the bottom of the pupa or wick the water onto the wings and a loose-fitting lid placed atop to create a humidity chamber. Fenestrated pupae are very quickly after injection sealed with a piece of #1.5 cover glass custom cut with a diamond knife to fit atop the wing in the pupal opening. This piece of glass is subsequently adhered into place using silicon surgical glue (Kwik-Sil) applied using the head of an insect pin. All pupae stained with non-nuclear stains are given at least an hour after injection for dissemination and binding of the stain, with the Spirochrome SiR-type cytoskeletal stains looking best starting around 2.5-3 hours incubation time. Nuclear stains are imaged as soon as possible after injection to not miss any epithelial divisions. Thirty minutes prior to the onset of imaging, wing flipped and fenestrated pupae are injected in the pleural membrane of abdominal segments III and IV with 2μL of 1mM tetrodotoxin citrate dissolved in distilled water using a Nanofil 100μL sub-microliter syringe outfitted with a metal 35G needle (World Precision Instruments) to prevent movement during imaging.

I.2.4 Imaging and microscopy

Time lapses of pupal development were captured using a ZEISS Axio Zoom.V16 with an Axiocam 512 color camera. Procedural photos and developed wings were imaged using a Samsung Galaxy S20 5G rear-facing camera, aided by a ZEISS Stemi 508 dissecting microscope with ZEISS Apochromat 1.5X FWD 53mm magnification and ZEISS 16X 14 Br. foc. eyepieces.

Two-photon imaging of nuclear divisions was conducted on a ZEISS LSM710 inverted confocal with Coherent Chameleon Vision II laser and 20X 0.8 M27 objective and Non-Descanned Detector (NDD1) consisting of a two-channel reflected light GaAsP detector. A laser wavelength of 722nm was used for two-photon excitation of Hoechst33342 dye at ~2-4% laser power.

Movies of the actin cytoskeleton were acquired with a ZEISS LSM880 Airyscan inverted confocal outfitted with the following ZEISS objectives: 5x Fluar 0.25NA, Plan-Apochromat 10X 0.45NA, Plan-Apochromat 20X 0.8NA, C-Apochromat 40X 1.2NA water. The Airyscan detector was configured for 'SR' (super-resolution) in FastAiryscan mode with Cy5 filter settings. The 633nm laser was set at a total output of ~22% in FastAiryscan mode for far red SiR-Actin stains with an optical zoom between 1.8x and 3-4x for wing sections and individual scales, respectively. For recording cytoskeletal dynamics in Airyscan mode, laser power should not exceed 3%, especially in early scale growth, lest malformations of the actin cytoskeleton manifest. FigI.10 and FigI.11 were imaged using a ZEISS LSM980 Airyscan 2 inverted confocal with ZEISS C-Apochromat 40X 1.20NA water FCS and Plan-Apochromat 63X 1.40NA oil DIC M27 objectives, respectively, with GaAsP-Pmt detectors on Airyscan mode MPLX SR-8Y:3.7. The laser power used for exciting organelle and microtubule dyes was kept from 2-4.5%, depending on strength of signal and Cy5 filter sets used for SiR-Tubulin. For all confocal imaging, pixel dwell time was found to be an important parameter for ensuring the capture of normal cell dynamics. A

unidirectional scan speed with no averaging was always used. For fast acquisitions, a pixel dwell of around .6µsec was selected and for scans with 3min and 8min intervals, a pixel dwell not exceeding ~1.3µsec was targeted. Z-sections were selected to accommodate the entire growing scale and spaced optimally for acquisitions as calculated by Zen software, save for Fig.I.12 where 1µm sections were selected to reduce laser exposure.

STED microscopy of emerging scale actin networks was conducted on a Leica SP8 Stellaris inverted confocal with an HC PL APO CS2 100X 1.40NA oil objective and Power HyD detector. The depletion line was run at 775nm at 1.28W for 633nm excitation of SiR-Actin dye and acquisitions taken at ~400 bidirectional scan speed over ~20 z-sections, no line averaging, line accumulation 12, and magnification 100. Laser power was adjusted throughout the acquisition, starting at .8% and finishing at 1.8% to compensate for minor fluorophore bleaching after 10hrs.

As all imaging was undertaken on inverted microscope configurations, slide-mounted diamondback moths and fenestrated pupae required inversion to be imaged. Wing flipped pupae are already mounted in glass bottom dishes and so require minimal adjustments for image acquisitions from inverted set-ups. Slide-mounted *P. xylostella* are, too, simply flipped and placed in a standard slide holder mount, but fenestrated pupae must have clay feet added to the ends of their mounted slide to adjust the height of the pupal opening to be level with the base of the slide mount. This step is crucial to ensure the lowest possible laser exposure as any tilt imposed by the clay mounts will require additional Z-frames to compensate; though if the pupal wing itself is tilted within the fenestrated pupa, the clay can be adjusted to offset this and achieve a level plane of imaging.

I.2.5 Image processing and scale parameter measurements

Image acquisitions were saved as .TIF hyperstacks and primarily processed using a combination of Fiji opensource software and Imaris (Oxford Instruments) (Schindelin 2012). All Airyscan movies were processed using the Airyscan Processing function available in Zen software, using default settings. In Fiji, nuclear, vesicle, and cytoskeletal movies were adjusted or enhanced for contrast depending on signal using the Auto setting or Enhance Contrast set to 0.35% saturated pixels, respectively, and normalized across the time series. Scale bars and time stamps were added using the Scale Bar and Time Stamper functions. If significant movement occurred during acquisition or late-stage morphogenesis shifted focal structures from central frame, Correct 3D drift was run to align frames (Parslow *et al*. 2014). Frames that experienced too much movement and did not have discernible morphology were removed from the movie file after timestamps had been added to keep a proper morphological timeline. Frames above the wing membrane or developing scale tips and below the nuclei/scale bases, for nuclear and cytoskeletal movies accordingly, were manually removed to reduce noise in projected views, making sure not to clip any sections of scales or relevant cells. Frames containing patches of autofluorescence from overlying cuticle, usually as a result of the mounting having been tilted, had these areas manually excised and replaced with black space. Once a stack was trimmed, maximum intensity or standard deviation intensity projections were used to better visualize development. Nuclear lineage movies had a 1.25σ Gaussian blur applied to aid visual particle tracking. When a three-dimensional projection was necessary to resolve dynamics, image stacks were oriented in Imaris,

then saved as a new .TIF stack at the proper orientation to be exported back to Fiji for annotation and data collection.

To visually trace lineages of nuclear divisions, representative nuclei demonstrating different division regimes were manually cropped from drift-corrected movies and the focal nucleus centered using a custom landmark-based registration plugin called Manual_Registration.py that aligns features based on user-selected ROIs (Sup.I.3; Sup.I.4; & Sup.I.5) (Eglinger 2018). The focal nucleus was outlined with an ROI for all timepoints in a movie with the desired nuclear LUT. This selection was then copied into a greyscale version of the same movie to leave only the desired elements in color. To assess changes in actin structure throughout scale elongation, the STED movie of scale growth was used to measure the width of actin rods as it had the greatest resolution of early filaments. For each time point, a line segment was drawn normal to the long axis of every filament that could be discerned across the Z-stack. As not every filament could be followed throughout all time points, these values were averaged per acquisition frame and graphed as a function of developmental time along with their standard deviation. To record changes in scale shape over time, the STED movie was projected using Imaris such that their cross-sections were apparent. As the movie was registered, this cross section should represent a single, consistent slice through approximately the same area of the scales throughout development. In Fiji, ROIs were drawn to outline the outside border of cover (n=3), middle (n=3), and ground (n=3) scales for each serial acquisition and their length, width, and aspect ratio obtained using Multimeasure. These values were graphed as a function of developmental time using the built-in Fiji graphing software for both all individual scale measurements and the average values for each scale type. Curves were imposed to fit the distributions of points post-hoc, with an exponential function with offset determined to approximate the developmental trajectories (App.I.4; App.I.5). It should be emphasized that these curves were not calculated to optimize fit based on model selection algorithms but were purely fit by eye to give a sense of the relative patterns and timings of growth between different scale types.

Annotations and outlines for movies were made using Fiji and figures were compiled using Illustrator (Adobe Creative Cloud). Montages were extracted from movies using Make Montage in Fiji with a scale factor close to 1 to maintain resolution and an appropriate number of panels to demonstrate the target morphogenesis.

I.2.6 FRAP

P. xylostella pupae were prepared for Fluorescence Recovery After Photobleaching (FRAP) through staining with just SiR-Actin dye ~16-24hrs after scales had begun to grow. Starting imaging settings were used as for a typical actin imaging session, as outlined above, and FRAP was performed by selecting a scale that was roughly flat and could fit mostly into a single Z-slice at 4x zoom. Either the distal tip (n=4) or the scale interior (n=3) were then centered in frame and the Zoom set to 24x with the laser increased to 100% output until the fluorescent signal in frame completely faded. Immediately after, the laser was returned to the prior power and zoom adjusted back to 4x so an acquisition could be taken of the selected single Z-frame at 1sec intervals until fluorescence was recovered or 10min had transpired. Resultant movies were registered in Fiji with Fast4Dreg and Enhance Contrast>Normalize Intensity run with 0% saturated pixels (Laine

et al. 2019). Regions of interest (ROIs) of equal size were drawn overtop a non-photobleached rod as a positive control, outside the scale as a negative control, and inside the photobleached region along the actin rods. The average intensities of these regions were then measured using Multimeasure. Intensity values for each ROI were plotted using the built-in ImageJ graphing tool and lines of best fit calculated in the same tool to fit each distribution (App.I.1; App.I.2; App.I.3).

I.3 Results

I.3.1 Lineages of Sensory Organ Precursors vary within and across species

Conflicting accounts exist in the literature regarding the cell lineage of Sensory Organ Precursors in lepidopteran wings. The death of the pIIb cell, that normally gives rise to neuron and sheath cells, in wing scales of *Ephestia kuhniella* was discovered through serial sectioning and since dissections of developing *Junonia coenia* wings and label-free live imaging of *Vanessa cardui* have recorded this apoptosis, as well (Stossberg 1938; Galant 1998; McDougal *et al*. 2021). However, other researchers do not corroborate these findings, for instance recording no division of enlarged SOPs in *J. coenia* from their independent serial dissection series (Nijhout 1980). Reviewing the original evidence, it is apparent that in most cases there lacks convincing thorough effort to resolve these lineages outside *E. kuhniella*, as most apoptosing cells can just as easily be misinterpreted epithelia or the images are lacking in resolution to definitively state a cell division took place. Staining the wing with Hoechst33342 unambiguously labels the nuclei and makes documenting mitotic events simple through counting the number of spindles a cell creates. Live imaging intrinsically avoids the issue of having to attempt to age-match wings from a dissection series, and, furthermore, the lack of background that results from specifically labeling the DNA—and therefore chromosomes—with Hoechst33342 makes following individual cells throughout development much simpler than label-free methods.

I observed the early differentiation of wings using Hoechst33342 staining to visualize nuclei across two moth and one butterfly species to determine whether or not a pIIb cell is produced and undergoes apoptosis prior to the division of the pIIa cell into the scale and socket. From these movies of nuclear divisions (*J. coenia* n =11, *P. xylostella* n=4, *T. ni* n = 1; one of each represented in Sup.I.3-5), all nuclei staying within the imaging frame throughout the timelapse duration were visually tracked to follow their divisions and document the variation in cell lineages. A typical movie produced approximately thirty nuclei that fulfilled these criteria, and these SOP cells form the bases of the observed lineages. Lineages of representative nuclei from this set were then compiled to showcase the variation in nuclear divisions documented in these observations (Fig.I.2). *T. ni* SOPs were found to follow the canonical set of divisions described in *E. kuhniella*, with two obvious mitotic spindles forming and a degenerating pIIb appearing after the first (Sup.I.4). By contrast, *J. coenia* SOPs appeared to undergo a singular complete division to form scale and socket daughters, seemingly foregoing a first division. Instead of a mitosis, the SOP nucleus appears to undergo partial DNA condensation, failing to form a metaphase plate, and then repositions itself without producing a degenerating pIIb daughter; subsequently, it settles into the epithelium and divides into the scale and socket (Sup.I.5). Straddling these two extremes, *P. xylostella* SOPs appear to be capable of either undergoing a

pIIb division or not, with different nuclei either displaying two canonical mitoses to produce a degenerating daughter cell after the first, or a phenomenon reminiscent of *J. coenia* in which a first spindle seems to be initiated, aborted without producing an asymmetric daughter, then followed by a normal mitosis into scale and socket cells (Sup.I.3). In the diamondback moth, this second strategy whereby an apoptosing cell is not produced after the first condensation event is quite less common than the canonical production of the pIIb, having only been observed in around five nuclei. A delayed release of the pIIb cell after the initial condensation may obfuscate the ability to document and track its production in the cases of these seemingly non-canonical lineage schemes. And so, further investigation with higher resolution is needed to resolve this apparent discrepancy. Regardless, in all cases where an apoptosing cell is definitively produced, it is many times smaller than the persisting cell and it very quickly delaminates from the epithelial layer and is whisked away with the flow of hemolymph over the wing.

I.3.2 Actin organization follows discrete phases in early scale growth

It is clear from serial dissections of phalloidin-stained developing scales that the actin cytoskeleton plays a large role in templating macrochaete structural elements, but a standing question remains as to how extremely fine filaments present in initially projecting scale buds give rise to the parallel, thick 'rods' running the length of mature scales that prefigure the placement of ridges (Dinwiddie 2014). I stained *P. xylostella* and *J. coenia* wings with SiR-Act to track fine and bundled actin filaments across scale development, focusing on the first 12 hours starting from the onset of elongation. Using lower magnification objectives to observe dynamics across a large wing area, scales were found to initiate elongation at different times but elongate at roughly the same rate, with scales along the margin starting to grow first, followed by cover and sensory scales, middle scales, and, finally, ground scales (Fig.I.3).

Inspecting individual scale trajectories, discrete phases of growth can be categorized: 1) initial projection, 2) first extension, 3) cylindrical elongation, and 4) flattening (Fig.I.4). In the first phase, a mound-like scale filled with a cloud of actin filaments emerges from the wing membrane before quickly transitioning to a slightly widened peg that defines the second phase. Continued growth of this peg without widening typifies the third phase which secedes to the flattening phase when this cylindrical extension quickly widens and spreads along its distal boundary like a Chinese fan.

To understand how actin filaments are being rearranged throughout these stages it is necessary to increase the resolution of acquisition; to this end, STED microscopy provides increased resolution through elimination of out of focus light, under optimal conditions to the extent of going beyond the diffraction limit (Hell & Wichmann 1994). Since the distance between developing scales and the coverslip is widened due to the presence of hemolymph, peripodial membrane, and pupal cuticle, STED microscopy is not able to easily provide nanometer scale resolution of developing scales, but it does provide greater discernment of actin in initial projection stages. Actin rods were found to be approximately 25% wider at the initial projection and early first extension stages compared to cylindrical elongation-stage filaments, experiencing a drop in width as the first extension stage progresses (Fig.I.5). This loss in bulk is only gradually regained when the actin rods become continually wider as the scale flattens. Although

these stages were excluded from the data collection, from other movies it is apparent that eventually a stable actin rod width is reached and widening halts.

Close inspection of adult wing scales reveals cases where parallel ridges appear to bifurcate, presumably due to the apparent fusion of adjacent ridges. Observing mature actin rods in scales around 24hrs after elongation, instances of "intercepting rods" can be found (Fig.I.6). These rods grow between two rods that already stretch basally to apically from the stalk to the scale tip and rapidly, on the order of seconds, contact and fuse with, then separate from their adjacent actin rod neighbors. After each contact and fusion event, the intercepting rod elongates apically a short distance again and repeats the process, inching along toward the distal tip of the scale.

I.3.3 Distal actin growth precedes interior bundling into rods

Simple observations give a new perspective and, with it, hypotheses about how growth is being actuated, but a major boon of live imaging is the potential to introduce manipulations and in real time observe the effects they have on morphogenesis and physiology. It is not definitively discernible from the previous observations of scale growth alone from which region of the scale elongation occurs. Growth could be biased and the actin rods grow from their basal ends near the scale base, pushing the older rod sections and cell membrane forward together, or they could grow from their distal ends and directly push against the cell membrane itself. Alternatively, actin rod extension could be unbiased and occur throughout their length, with extension proceeding in a manner akin to nested telescoping poles. Since actin elongation in these experiments is visualized using a fluorescent marker available in excess in the wing hemolymph that can continually bind to actin monomers as they are added during the growth process, by abrogating fluorescence of bound marker-tagged actin through photobleaching it is possible to observe the manner in which new, non-photobleached, actin monomers bind. Photobleaching different regions of the scale can determine if there is heterogeneity throughout the cell body in the rate at which actin recovers and looking at the directionality of recovery along an actin rod can suggest how processes like polymerization and bundling proceed. When the distal tip of the scale is photobleached, fluorescence rapidly recovers within two minutes in a biased proximal-to-distal direction. By contrast, a photobleached central section of a scale—far removed from the distal edge—experiences a tremendously slow recovery, not achieving starting intensity values even after ten minutes, with no bias in direction of recovery (Fig.I.7).

I.3.4 Differential growth of scale types

Scales are heterogeneous in the sense that different scale types exist next to each other in patches but also in specific areas of the wing. These unique forms are the result of different dynamics during growth, which can be captured using live imaging to compare morphological trajectories. By looking at the developmental process and final morphology, it is possible to differentiate scale types. Middle scales are almost two times thinner than either ground or cover scales, and despite having a similar final aspect ratio, ground and cover scales take different paths in reaching their ultimate shape (Fig.I.8). By imposing exponential curves with offsets onto the data, it is possible to estimate the point where scales transition to the flattening stage by looking at the inflection points. By this metric, on average, cover scales begin flattening first, followed by middle, and then ground scales. It is important to reiterate that scales do not empirically

transition so smoothly as these curves might suggest, however. Individual scales seem to subtly oscillate in aspect ratio, fluctuating between slightly higher and lower values as the average value nevertheless increases over time (Sup.I.6).

Specialized scales can have even more divergent shapes, and accordingly vary with regard to their growth. “Cilia” or androconial scales in *J. coenia* resemble bristles and like these sister macrochaetae maintain a circular cross section throughout their growth (Sup.I.7). They also are the first macrochaetae to grow in the wing interior along with sensory scales, elongating rapidly well before adjacent ground and cover scales, and at approximately the same time as the marginal scales that border the wing (Fig.I.9).

I.3.5 Mitochondrial networks and vesicle trafficking

The rapid growth and giant size of scale cells indicate that they undergo significant metabolism and internal signaling. Mitochondria are highly dynamic along the scale body, and particularly at the tip (Fig.I.10). Overall, these organelles adopt a reticulated configuration characterized by rapid fusion and segregation of adjacent mitochondrial arms as the scale continues to grow. The greatest concentration of mitochondria appears to be within the core of the scale. As the scale volume increases with progressive widening, the mitochondrial reticula become more diffuse as it approaches the scale margins and seems to adopt a stringier morphology.

Vesicle trafficking, too, is highly dynamic along microtubule highways within the scale cell body (Fig.I.11). Individual particles tracked at second intervals followed microtubule routes along the curved distal tip of the scale edge and were shunted back and forth within the scale center and midline. From these observations, it seems there are particular routes, or at least paths of least resistance, within the scale for transport that roughly follow up the center of the scale to the tip and then out and back along the lateral edges to the pedicel. To this point, particles seem to move faster as their distance from the midline increases. This may be an attribute of the dynamics of motion specific to each area. Particles along the scale midline seem to move jerkily, advancing distally some distance then sliding back in the proximal direction compared to the circuitous, but almost always forward, path of vesicles along the margins. Performing a maximum intensity projection, it is possible to observe the distribution of vesicles within the scales. Apart from the aforementioned small vesicles in the scale periphery and tip, two large vesicles occupy the medial base of each scale at the junction between the shaft and pedicel. Viewed live, these larger spheres are nearly stationary as smaller vesicle bodies similar to those at the tip orbit and sometimes fuse with its mass.

I.3.6 Actin remodeling in Papilio palinurus *scales undergoes two stages*

Modifications of the actin cytoskeleton that correlate to features of structural coloration are a black box when attempting to understand how such complicated structures evolved. *P. palinurus* possess modified cover scales on their dorsal fore- and hindwings with dimple arrays on their abwing surface that structurally mix reflected yellow wavelengths from their center and retroreflected blue wavelengths from their walls to appear green to the eye (Vukusic *et al*. 2000). These dimple arrays are known to be prefigured by actin arranged in a hexagonally arranged honeycomb-like pattern, in a similar manner to how ridges follow templating by parallel actin

rods. It has already been documented that these scales begin development typically with normally spaced parallel actin rods and that at some point during late scale development alternate actin rods disappear and shortly after the hexagonal array arises (Null 2017). By watching these scales develop live, it is possible to appreciate how rapidly these transitions occur, with each taking only 45min to 1hr to transpire. In the first step, every other actin rod breaks down and disseminates along the cell cortex leaving only the alternate rods behind. Next, approximately two hours after, actin bridges begin to appear between the alternate rods, ostensibly from condensations of the cortical actin, and these within minutes arrange into the hallmark hexagonal networks. Actin signal along the initial hexagons is weak before becoming progressively stronger in subsequent timepoints. This second stage is also extremely sensitive to laser light, with too high or too long an exposure disrupting the fine filamentous actin and preventing array assembly. As such, to capture this stage, the movie was timed to precisely begin at the onset of the transition and acquisitions were only acquired in AiryscanFast mode. Interestingly, by contrast, the first transition displayed only the usual sensitivity to laser light expected for scale actin staining.

I.4 Discussion

The live imaging techniques developed in this account captured cellular phenomena that would be particularly onerous or impossible to record from fixed samples. The ability to track multiple epithelial nuclei throughout early wing development and specification of sensory organ precursors demonstrated how a uniform epithelial sheet becomes organized. The flexibility to investigate this question across two moth and one butterfly species unveiled key differences in cellular arrangement and division, in particular. Although sensory organ precursor cells in *P. xylostella* and *T. ni* are specified first in rosettes, just like has been recorded for papilionoids like *J. coenia*, they were not found to undergo the subsequent arrangement into obvious parallel rows of SOPs before pIIa mitosis that is considered a hallmark of butterfly wing organization (Yoshida & Aoki 1989). Instead, scale-socket pairs remain roughly in the position they were in when they divided. This difference can be attributed to the fact that moths display three scale types, ground, middle, and cover, whereas butterflies possess only the former- and lattermost. With three scale classes, the moth wing scales repeat along iterating triangles, as can be seen in the emergence sequence of scales in Fig.I.3. Consequently, to prefigure this arrangement, the SOPs have an interleaving, staggered pattern as opposed to being shuffled into parallel rows.

Long processes extending from the basal surfaces of wing epithelial cells have been implicated in coordinating tissue rearrangement and cell spacing in *Manduca sexta* wings (Nardi & Magee-Williams 1986). Live imaging could help map the development of these networks throughout wing morphogenesis across a range of species representing scales arranged from rosettes to rows. Additional layering of this data with force-linkage modeling and clade-specific evolution of cell surface receptors involved in cell-cell junctions could provide an integrated view of the actors and mechanisms responsible for the evolution of scale spacing relationships overall (Nijhout & McKenna 2018).

In general, scale arrangements in lepidopteran wings have been described as a sequence where a uniform scale type arranged randomly in the earliest branching lineages is followed by the differentiation of scale types and finally the patterning into rows of two types of scale in Ditrysia (Simonsen 2001). This observational data of moth SOP arrangement and scale growth raises an eyebrow to this notion of "random" and gives additional criteria that may be useful in differentiating morphologically similar scales. The random arrangement described for early branching moth clades may actually be a non-standard array of cryptic scale types that can only be differentiated upon watching them emerge. In such a case, even if they are largely identical or indistinguishable in the adult wing, emergence time itself could be used as a criterion to state that scales belong to the same class. Observing SOP specification and scale emergence periods for early branching lepidopteran lineages could aid in explaining the origin of scale types writ large by revealing the ancestral networks of specification that have been modified or elaborated in later-branching moths and butterflies to differentiate individual classes.

Tracking divisions of sensory organ precursors across different species uncovered undescribed heterogeneity in nuclear lineages and resolved differing accounts of these developmental trajectories in the literature. pIIb cells were only found to be produced consistently in *T. ni*, with a proportion of SOPs not producing any apoptosing daughter cells despite seeming to undergo two mitoses in *P. xylostella* and SOPs in *J. coenia* only forming one bona fide spindle and no apoptosing daughter cells when it divides into a scale and socket cell. In these instances where no apoptosing pIIb daughter cell is produced, there is always either a disorganized spindle formed or some major condensation of the nucleus (Fig.I.2D). Since the SOPs where this phenomenon was observed to occur form normal scale and socket arrangements and proceed to grow scales, these nuclear arrangements prior to the scale-socket split are interpreted as modifications or deviations from the canonical pIIb-producing lineage. The separation of pIIa and pIIb cells has been described as important for isolating neuron-inducing factors, and it may be that in these species other mechanisms exist to enlist a macrochaete program. The nuclear condensations or wonky spindles may serve to redistribute nuclear compartments in a manner that does not require cytokinesis. Diversification of SOP-derived structures through alterations of division patterns and daughter cells is a widespread phenomenon underpinning much of the diversity in sense organs, and a similar alteration may have been canalized in certain species of butterfly where pIIb separation is largely unnecessary for the majority of SOP cells (Lai & Orgogozo 2004). It is tempting to suggest that since even in observing canonical division patterns, the ephemeral nature of the jettisoning pIIb combined with its extremely asymmetrical division in *T. ni* and *P. xylostella* may indicate that the cell lineage may have evolved to be more economical or efficient, as modifications to the lineage that minimize the volume of hundreds of thousands of apoptosing cells for the wing scales may be advantageous metabolically. The wholesale elimination of pIIb cell separation, then, may spontaneously arise in certain lineages through either drift along this evolutionary trajectory or as a continuation of this adaptationalist logic. Regardless, the varying accounts of the SOP lineage in the literature can be explained as a product of two factors: 1) first divisions of the SOP, when they occur, happen extremely quickly and produce a highly ephemeral and transient pIIb daughter cell that quickly detaches from the

epithelial layer and 2) the production of a distinct pIIb daughter appears to be variable within and between species.

A fortuitous benefit of staining nuclei and observing SOP differentiation is also directly recording the polyploidization of trichogen nuclei after pIIa division and before scale extension (Sup.I.3). Nuclear size has been recorded to correlate with scale features such as overall length and suggested to play a role in determining cell class identity (Cho & Nijhout 2013; Iwata & Otaki 2016). Directly measuring endoreplication rate of trichogen nuclei and associating this value to the resultant scale may give additional support to these correlations which were formed using fixed pupal wings. This approach especially may reveal temporal differences in the initiation and cessation of polyploidization which could hint at possible influencing signals or response of different scale types. These correlation-based studies have also outlined proximal-distal gradients in nuclear size throughout different wing regions. An odd occasional outcome of pupal hindwings laid flat on a piece of Parafilm using the modified wing flip developed in this chapter is lack of cell death along the wing edge (Sup.I.2). Normally, the marginal epithelium of pupal wings undergoes apoptosis, presumably initiated by *cut* expression which prefigures the outline of the adult wing in the imaginal disc, but in these cases no such phenomenon occurs and the wing grows all the way to the edge of the peripodial sac (Macdonald *et al*. 2010). The wing margin patterns are similarly disrupted as the wing edge is thought to serve as a signaling center (Reed 2020). It would be very useful in understanding cell fate decisions that govern adult wing shape to work out the conditions to replicate these expanded margin phenotypes and use them to determine the specific factors emitted from the wing margin that may influence polyploidy level.

Through observations of developing scales, growth was classified into four general classes of 1) initial projection, 2) first extension, 3) cylindrical elongation, and 4) flattening. Fluorescence recovery after photobleaching experiments conducted in different regions of the scale were conducted to determine where polymerization was actively occurring throughout the cell body during the cylindrical elongation phase. After photobleaching the scale distal tip, fluorescence recovered within a few minutes in a proximal-to-distal direction. This fast recovery of fluorescence indicates that the distal tip region is a site of rapid actin polymerization. By contrast, interior regions of rods underwent a slow process of fluorescence recovery, not returning to previous fluorescence levels after nearly ten minutes. Opposite to the distal tip, this result indicates that, within the scale interior, actin rods have stabilized and turnover mediated by polymerization has slowed considerably; however, the gradual return of fluorescence and increasing width of these interior rod sections suggests that actin bundling is still taking place (Fig.I.7) (Tilney *et al*. 2003). The proximal-to-distal direction of fluorescence recovery at the distal tip suggests that bundling occurs in older sections of the actin rods. A model of growth at the tip consistent with this recovery pattern could therefore be described as follows. Older, more proximal, sections add new fluorescent monomers first as they are already in the bundling process. Following this, the scale elongates in the tip region through thin, projecting actin filaments. With this increase in length, distance from the cell membrane at the leading edge increases and subsequent sections turn over into the bundling program and recover their fluorescence as new monomers bind. The low fluorescent output of individual new monomers of

actin binding to the filaments at the tip make it difficult to visualize these additions next to the comparatively much stronger fluorescent signal of many monomers bundling onto older actin rod sections, but the biased recovery from the proximal to distal direction with the context of a bundling program for older segments of the rod suggests that actin is maturing proximally and thus elongating distally. By contrast, in the center of the scale, fluorescence recovery is uniform throughout the interior rod section, indicating there is a stable level of bundling that is reached after a specific time threshold and there is no significant gradient in age for any parts of this section, as is the case for the elongating distal tip. Overall, these results suggest that scales are growing in a way very similar to *Drosophila* bristles, where a gradient between active polymerization and length extension and bundling exists along the distal-to-proximal axis, with older actin filaments experiencing more bundling than newer ones (Tilney & DeRosier 2005). Unlike in *Drosophila*, there does not appear to be any obvious apposition or cementing of actin filament segments to produce the rods, instead, these cords of actin appear to continuously and smoothly elongate throughout the lifetime of the scale (Tilney *et al*. 2004). It is very likely that the same or homologous molecular actors, namely forked and fascin, are responsible for the bundling of actin along the growing rods, but functional tests would be required to verify this (Tilney *et al*. 2000).

Interestingly, although the width of actin rods continuously increases in the second half of the first extension stage due to bundling commencing in proximal regions of filaments, before this point actin filaments in the early scale bud appear to be comparatively wider (Fig.I.5). A possible explanation for this disjunction may be that, before initial projection, actin filaments are pre-bundled and that once the scale tip extends beyond the epithelial surface these bundles are deconstructed and the constituent filaments linked together to more rapidly begin elongation. Observing actin organization early in the SOP just prior to scale extension could test this hypothesis. The existence of actin rods outside just the first extension phase may be supported by the observation of intercepting rods later in scale growth (Fig.I.6). Delays in the growth of initial filaments or perhaps even a later initiation and serendipitous reinforced extension may explain the difference in size of these rods which seem to readily bind to their neighbors as they advance along the length of the scale. Their existence also suggests that factors encouraging actin polymerization are not restricted to the growing tip and still persist in the scale interior despite bundling predominantly occurring in this region. Furthermore, there does not appear to be significant maintenance machinery in the proximal scale that break down actin filaments to regulate the number of rods, as the intercepting rods seem to grow continuously at a steady rate despite a winding trajectory. Ultimately, since these rods begin growth late or were delayed in some way, it is not possible for them to all “catch up” to the distal edge and they settle bound to an adjacent rod and pattern a bifurcation in the abwing ridges which can be commonly seen as an aberration in adult wing scales.

Different scale classes were found to vary in their patterns of growth, mostly as a product of the differences in emergence time. By looking at the inflection points of imposed curves in a set of aspect ratio measurements of developing ground, middle, and cover scales over time, it is apparent that the first-erupting cells, the cover scales, initiate flattening first followed in

sequence by the middle and ground scales. This staggered growth, apart from being a potential artifact of differentiation processes, probably aids in the imbrication of the scales. If all scale types were to initiate growth at the same time and grow evenly, it would likely lead to improper tiling and tangles. Perhaps aiding in this end, middle scales adopt a flatter aspect ratio, which may produce an interlinking physical barrier separating the cover and ground scales (Fig.I.8). The individual fluctuations in aspect ratio amongst scales as they gradually flatten may indicate some degree of physical response and feedback to modifications of the cytoskeleton, though much more precise recordings and mechanical perturbations would do better to resolve these oscillations in value. Viewing across different scale types, namely bristle-like cilia scales in comparison to more typical flattened scales, it seems as though to attain this particular morphology, all that is necessary is to continue developing in the third phase of cylindrical elongation without initiating flattening (Fig.I.9; Sup.I.7). Differences in scale type may then result from heterochronic shifts in development, whereby later morphogenic events, mediated by activated gene networks or otherwise, are delayed or avoided entirely. Molecular probing of factors specifying different shapes and closer inspection of cytoskeletal elements may help resolve the particular factors underlying classes of scale types.

Examining other organelles in the process of development reveals the physiological state of these cells. Despite being very large and their polyploid nucleus resting far away below the adjacent wing membrane epithelial cells, scales undergo extensive remodeling and shape changes. To achieve this, scales are extremely metabolically active with an extensive mitochondrial network and active vesicle trafficking along microtubules to orchestrate proper growth (Fig.I.10; Fig.I.11). The reticulated nature of the connections between neighboring mitochondria that span the elongating scale body suggests a heightened metabolic capacity and ability to quickly transport ions between cellular compartments. Likewise, the dynamism of the network and frequent contact and mixing between proximate mitochondria implies heightened potential for communication, exchanging of metabolic products, and redistribution of resources among regions (Glancy *et al*. 2020). Ionic redistribution is a particularly interesting role for these mitochondria as shifts in ion concentration can contribute to morphogenesis in the form of local osmoregulatory swelling and signaling in the case of calcium ions for fast, long-distance relays of local cell state that do not rely on cytoskeletal networks or the transport of specific molecules (Ishida-Ishihara *et al*. 2020; Rizzutto *et al*. 2012). Calcium signaling has been recorded in *Junonia orithya* pupal wings as part of normal development and in response to damage (Ohno & Otaki 2015). In this case, the signaling was recorded across the epithelium in a wave pattern emanating from certain, presumably eyespot, regions, but as a principle this supports the potential for similar mechanisms at the single-cell level, too. Fluorescence sensors of ion state have been manufactured to produce optical readouts of charge and pH (Lock *et al*. 2015). Use of these chemicals with the outlined live imaging methods may uncover hidden organization beyond the layer of physical structures (Lavis 2021).

Still, a bulk of material is transported within vesicles throughout the scale body along cytoskeletal tracks, and these probably contain mRNAs, waste products, and secreted proteins to make the chitinous exoskeleton that will remain once the scale projection retracts at the end of

the pupal period. Although LysoTracker ostensibly labels acidic lysosomal vesicles, many vesicle types other than lysosomes are acidic and other molecular cargo like mRNA molecules have been found to hitchhike on lysosomes to reach specific cellular destinations (Paroutis *et al*. 2004; Liao *et al*. 2019). As such, vesicle trafficking movies are interpreted broadly to understand movements of the spectrum of transported molecules. The variable speeds in different cellular compartments suggest heterogeneous binding of protein actors that regulate the tempo of motor proteins in the form of adaptors, bound opposed motors, and post-translational modifiers of the microtubule tracks themselves, among other possibilities (Guedes-Dias & Holzbaur 2019). Biophysical properties of the microtubule network as a consequence of curvature and thickness, along with movements of the microtubules themselves, could further diverge transport dynamics (Nasrin *et al*. 2021; Kulić *et al*. 2008). The discovery of two large vesicles above the pedicel highlights the potential for live imaging to provide insights in physiological dynamics and organization, too, by visualizing structures that may be too fragile to be preserved in fixed tissues. Although their role is unknown, since these vesicles are known to be acidic as they stain with LysoTracker, they may serve as vacuole-like receptacles for metabolic waste products as lysosomes and peroxisomes contain acidic hydrolases (Cooper 2000). Giving credence to this hypothesized role, smaller vesicles were occasionally observed fusing with them (Locke 2003; Lang & Jahn 2002; Lawaczeck 1978). Altogether, these data suggest a model where the observed smaller lysosomes pick up waste products from the scale body as they transit around the cytoplasm on microtubules and then return to these large vesicles to fuse and deposit their collected contents. As a first step in validating this hypothesis, measuring and overlaying speeds onto microtubule geometries would provide a roadmap to understand at least the flow of vesicle transport and determine whether the midline-anterograde/margin-retrograde directional bias that seems to be apparent from movies quantifiably exists. These routes for individual lysosomes would also indicate whether they originate or end at either or both large vesicles at the scale base. Longer term imaging of vesicle dynamics throughout scale growth would also indicate when these dual vesicles begin to form and whether they grow over time as a result of repeated fusion of smaller elements.

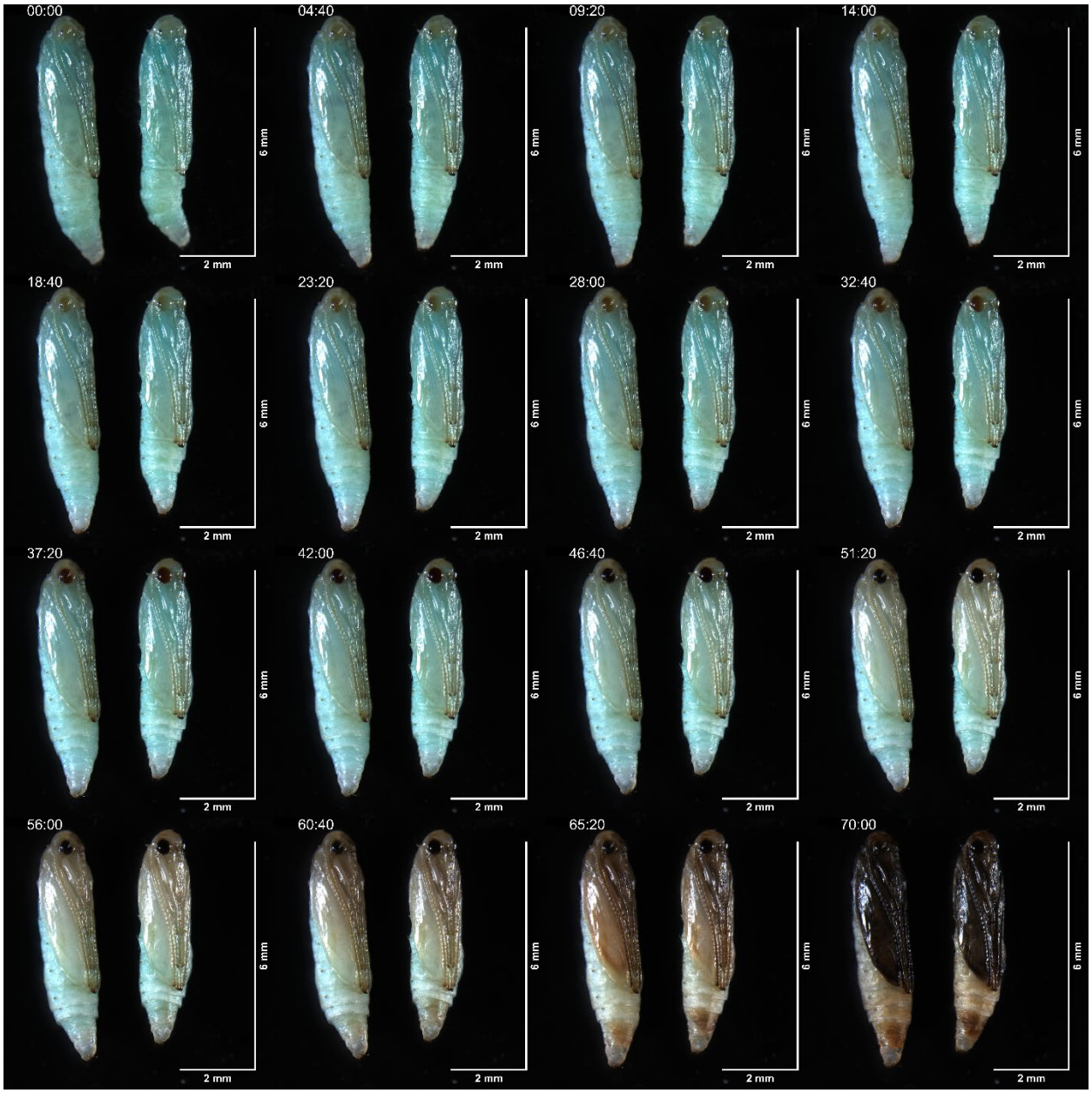
One of the most exciting ventures made possible by observing subcellular components during scale growth in a species-agnostic manner is investigation into the development of structural coloration. Looking holistically at the Lepidoptera, practically every feature of scales has been modified to interact with light in some way (Ghiradella 1991; Ingram & Parker 2008; Thayer & Patel 2023). Despite this extent of diversity in ultrastructure, it is likely that existing elements in ancestral scale types are modified, elaborated, or exaggerated to produce photonic elements (Parnell *et al*. 2018). Case in point, in the development of the dimple array in *P. palinurus* structurally green cover scales, despite the final configuration of a hexagonal mesh being a far cry from the parallel actin rods in a more canonical scale type, viewing the morphology ontogenically by watching its development live reveals two discrete morphological transitions that give rise to this derived condition (Fig.I.12). Each phase in this process involves actin reorganization, the first where actin is recycled from alternate rods back into the abwing cortex and the second where new crosslinks between the remaining ridges are manifested. Interestingly, this process of forming the derived structure builds directly off the ancestral construction. This

contingency reinforces the notion that scales have intrinsic stages of morphological transition that can be differentially targeted by evolution to produce diverse forms. In this case, the two phases occur at the point in development when an ancestral scale type restructures adwing actin to template microribs, lamellar elements, and future window regions (Lloyd *et al*. 2023 preprint). Morphogenetic modularity may have been the springboard for diversification of scale types and contributed to the variability of these cells within species and throughout lineages. Whether similar machinery is used to restructure actin in both the derived state and late stages of reorganization in canonical scales warrants further study. If so, modifications to the binding domains or differential localization of these machinery elements would become potentially important factors in tracking the evolution of this scale type.

I.5 Conclusions

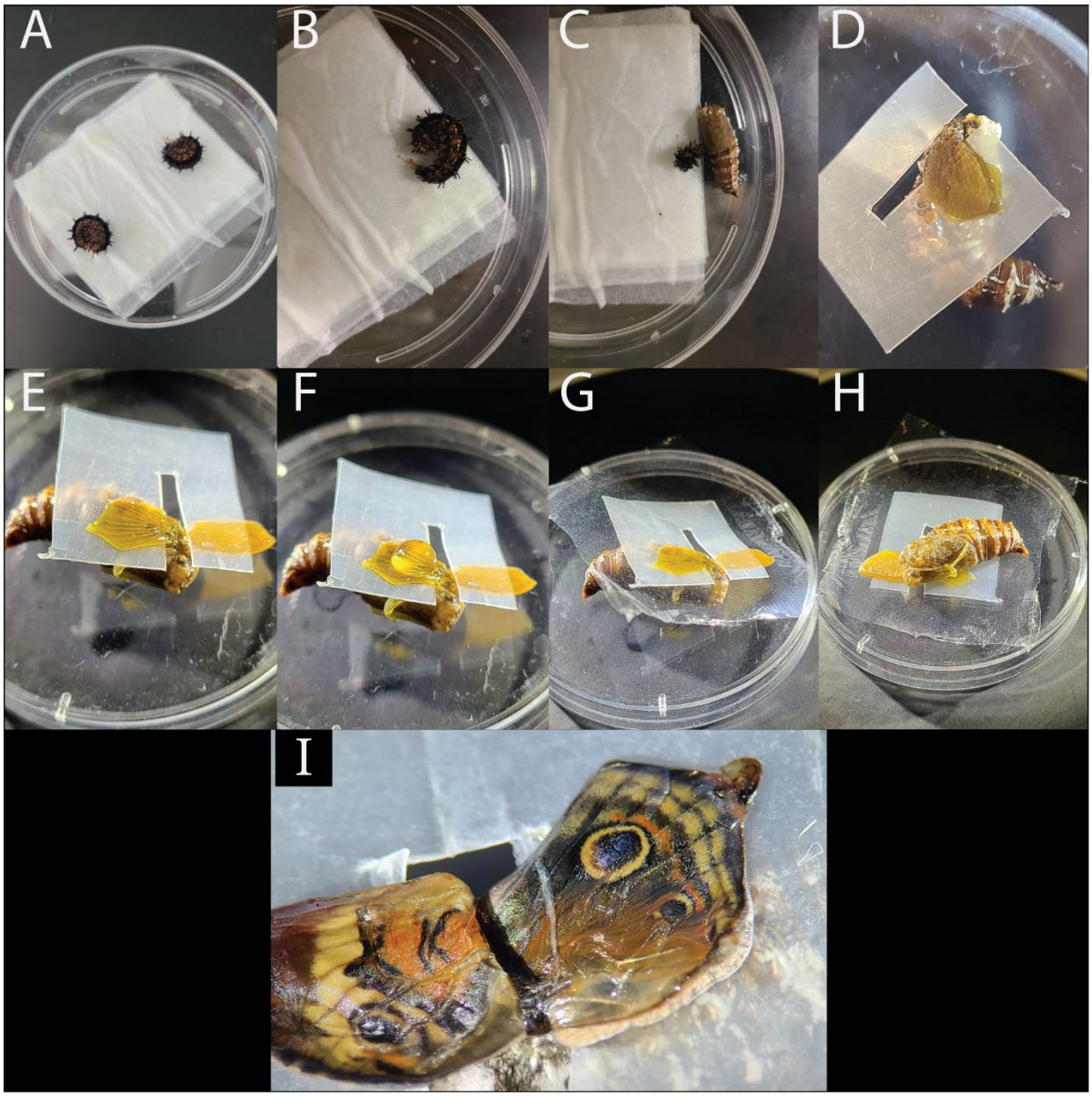
Scales as structurally complex, single cells provide a system with which to understand the evolution of morphological diversity. These methods provide the tools to expedite comparative studies of scale morphogenesis by lifting constraints that have limited our ability to characterize natural diversity. Using the modified protocols, any lepidopteran scale type that exists on the ventral forewing or hindwing can be subcellularly characterized throughout its ontogeny from a single individual. In testing the capabilities of this approach, scales and their generating mother cells were found to develop in discrete stages that differ between species principally in rate, period, or distribution of subcellular elements. This tweaking of common elements is likely the generating force behind scale diversity and explanatory for the range of morphological variation observed in instances of structural color.

Fig. I.1 Development of *Plutella xylostella*-a developmental system for scale growth



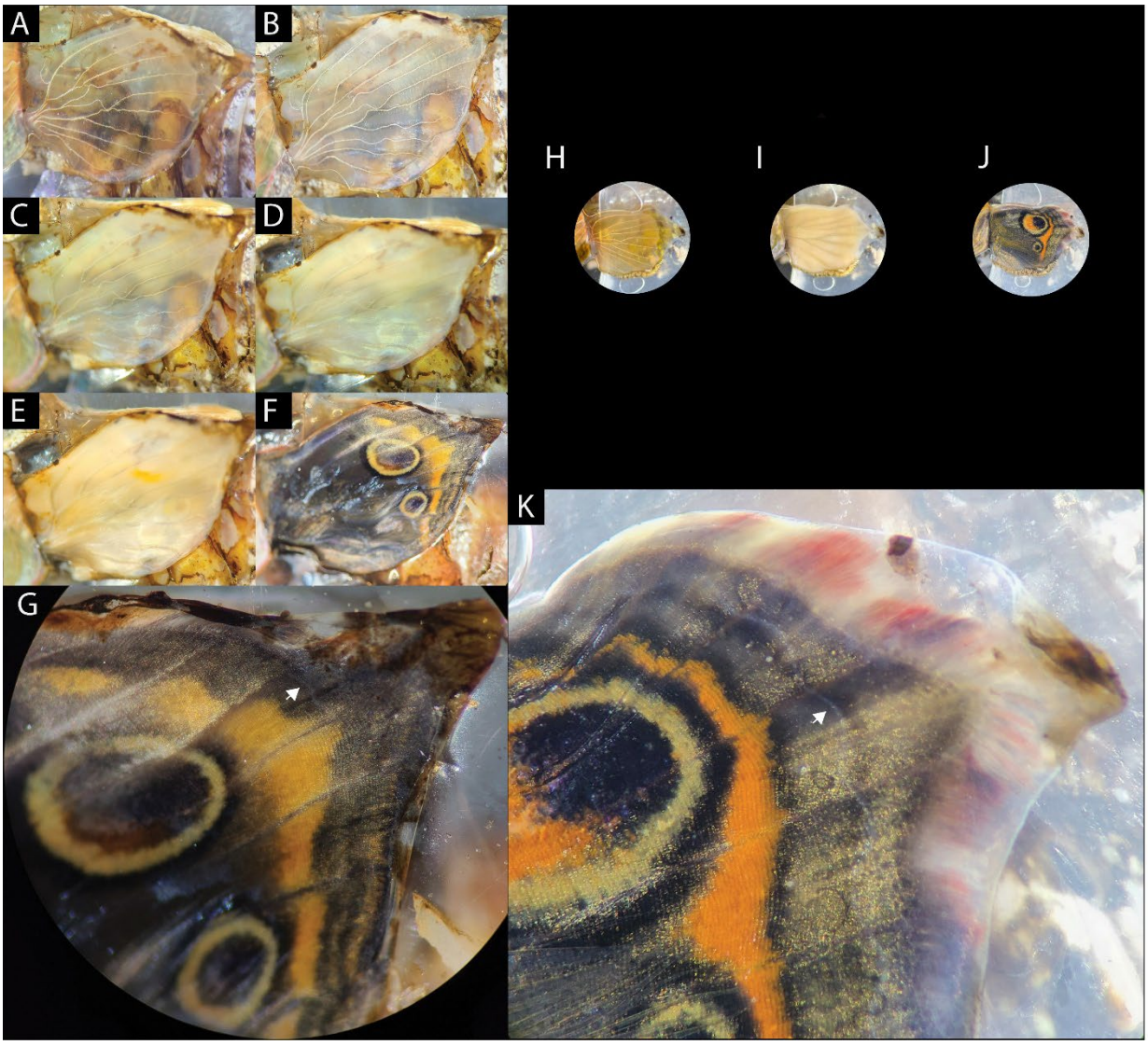
Development of the diamondback moth, *Plutella xylostella*, over a time scale relevant for scale growth: 24hr after pupation (00:00), when scales begin elongating, to 94hr after pupation (70:00), when pigments have been deposited and scale cell bodies start retracting from secreted cuticle. The scalebar measures 2mm x 6mm (width x height). Time is formatted in hr:min and images represent 4hr40min intervals sampled from a movie taken at 8min intervals. Dorsal is to the left and anterior is to the top of each frame. Note that the precise developmental stage can be assessed solely based on eye color.

Sup. I.1 Photographic protocol for modified Otaki *et al*. wing flip



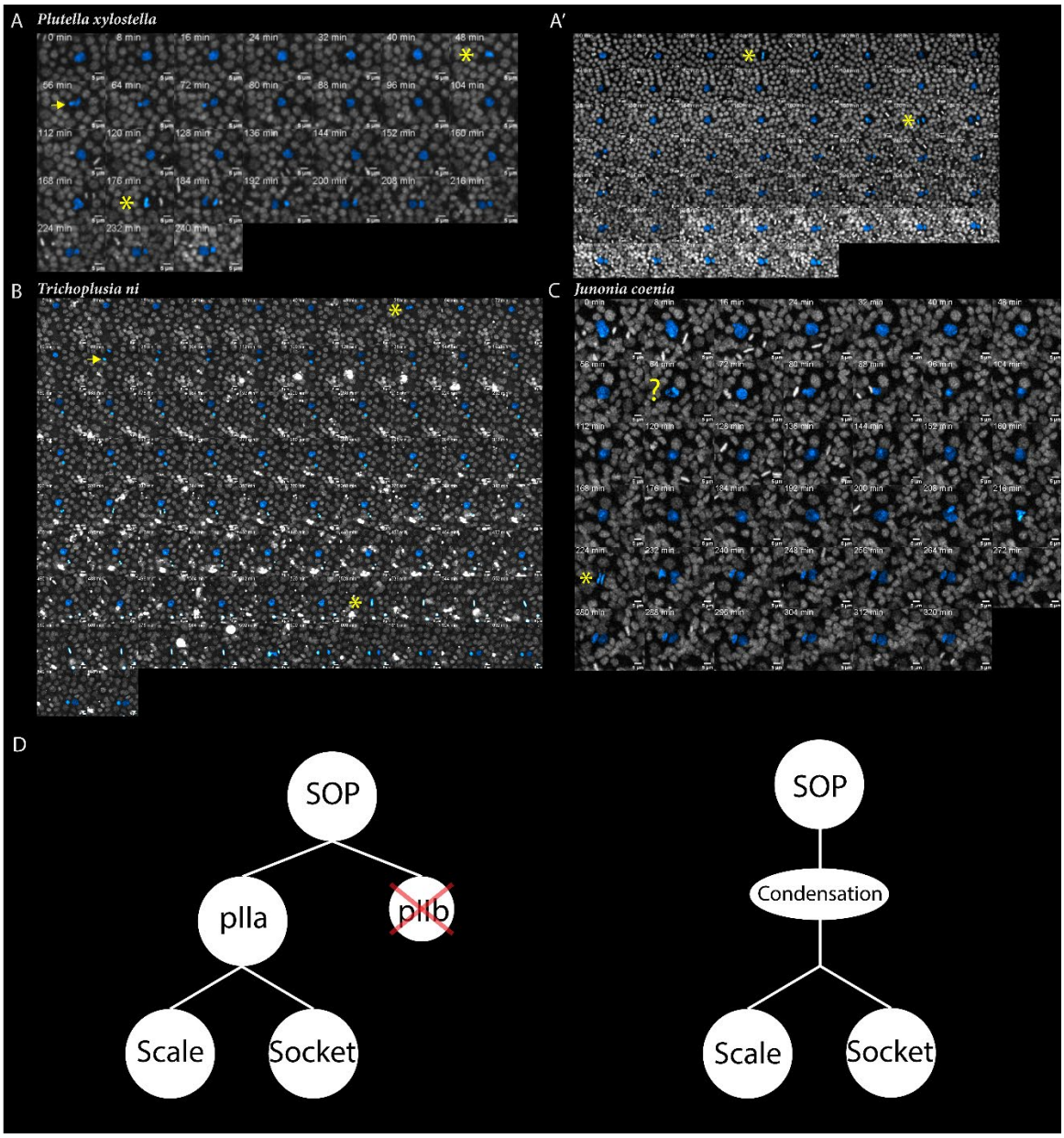
'J-shape' *Junonia coenia* pupae are isolated and placed in a moist environment made from a wetted Kimwipe and Petri dish (A) to observe them as they near pupation (B). Freshly molted pupae with the wings not yet fully elongated (C) undergo a "double" flip of both wings onto a Parafilm square with a custom-cut slit (D). The forewing is manipulated underneath the smaller slit lip (E) and a 5µL drop of Grace's:PTU:A+A solution is placed onto the hindwing surface (F). A square of sanitized Saran wrap is cut and placed over the hindwing (G) and the entire pupa is flipped (H) to leave to develop in a humid container. Approximately 5-6 days later, all color patterns appear on both wing surfaces of the flat-mounted pupa (I) (Note: some areas near the hinge can be prone to cut circulation of hemolymph flow and fail to develop fully as seen on the posterior hindwing margin of this individual).

Sup. I.2 Wingflip procedures occasionally show expanded wing margin phenotypes



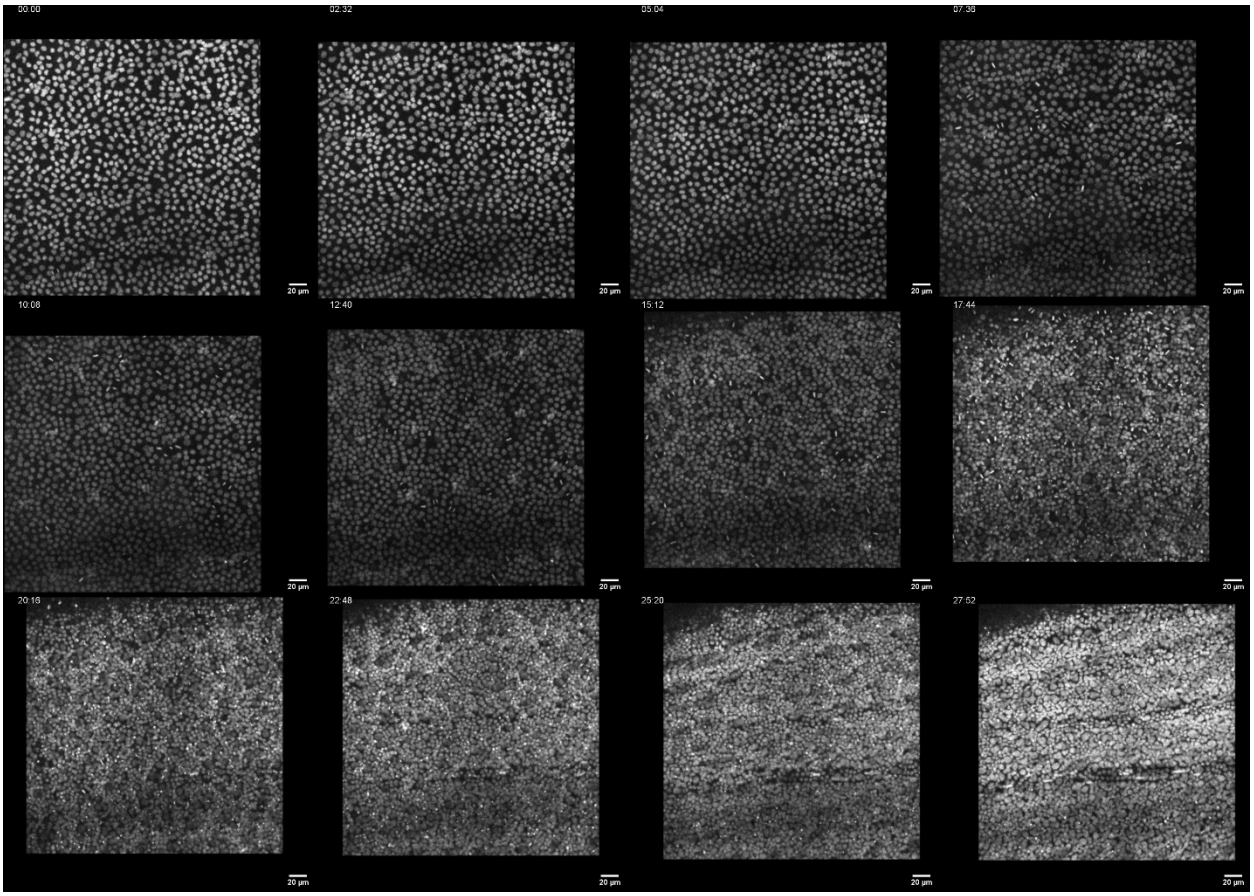
Case examples of two *Junonia coenia* pupae (A-G, H-K) that displayed expanded wing edge phenotypes after undergoing the modified wing flip procedure (Fig.I.1). A-F represent day intervals and H-J represent every-other-day intervals of wing growth, with A and H representing day 0, immediately after the wing flip was completed. G and K show magnified views of the marginal area of the fully patterned wings, with white arrows indicating a faint line where the wing edge would normally present. Wings oriented with distal pointing toward the right of the frame.

Fig. I.2 Sensory organ precursor cell lineages tracked across three lepidopteran species



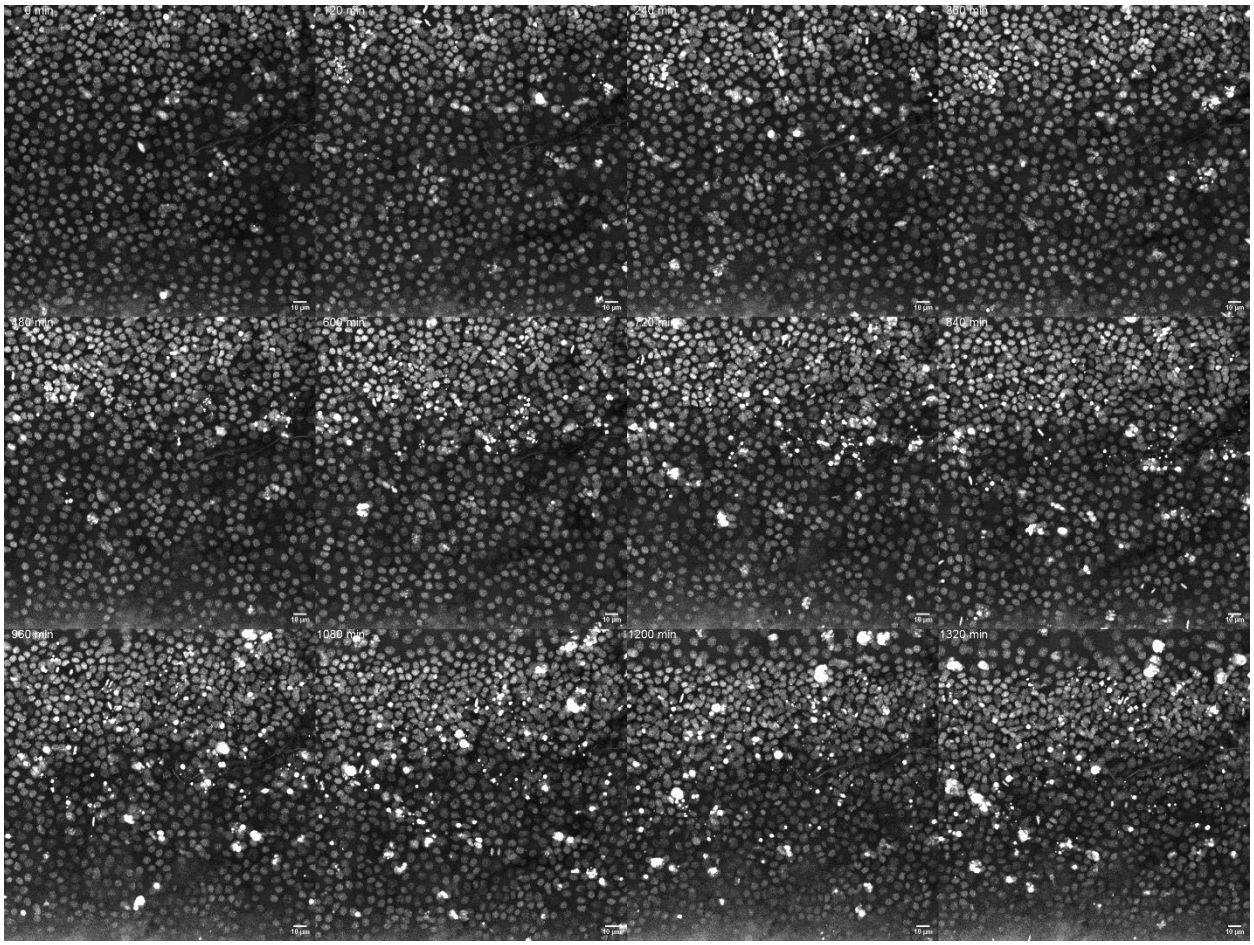
Two-photon confocal montages of maximum intensity projections of sensory organ precursor cell nuclear divisions in three lepidopteran species labeled with Hoechst33342: *Plutella xylostella* (A, A'), *Trichoplusia ni* (B), and *Junonia coenia* (C). Focal nuclei are false colored in cyan, with yellow asterisks marking time points when mitotic spindles form; uncertain divisions, as in (C), are marked with a yellow question mark. Degenerating pIIb cells are marked by yellow arrowheads in the first time point where they appear. Consecutive montage boxes represent timepoints taken at 8min intervals where the first tracking timepoint is designated '0min'. Tracking timepoints were sampled from longer movies documenting nuclear divisions before scale growth initiates, (A, A' see Sup.I.3, B see Sup.I.4, C see Sup.I.5). Scale bars measure 5µm. **D-** Schematics of observed cell lineage patterns. On the left, the canonical production of an apoptosing (red 'X') pIIb cell from an SOP mother is displayed, as observed in A and B. On the right, the SOP nucleus does not produce an apoptosing pIIb cell but instead condenses for a moment before undergoing a subsequent division directly into scale and socket daughter cells, as observed in A' and C.

Sup. I.3 Two-photon imaging of *Plutella xylostella* nuclear divisions



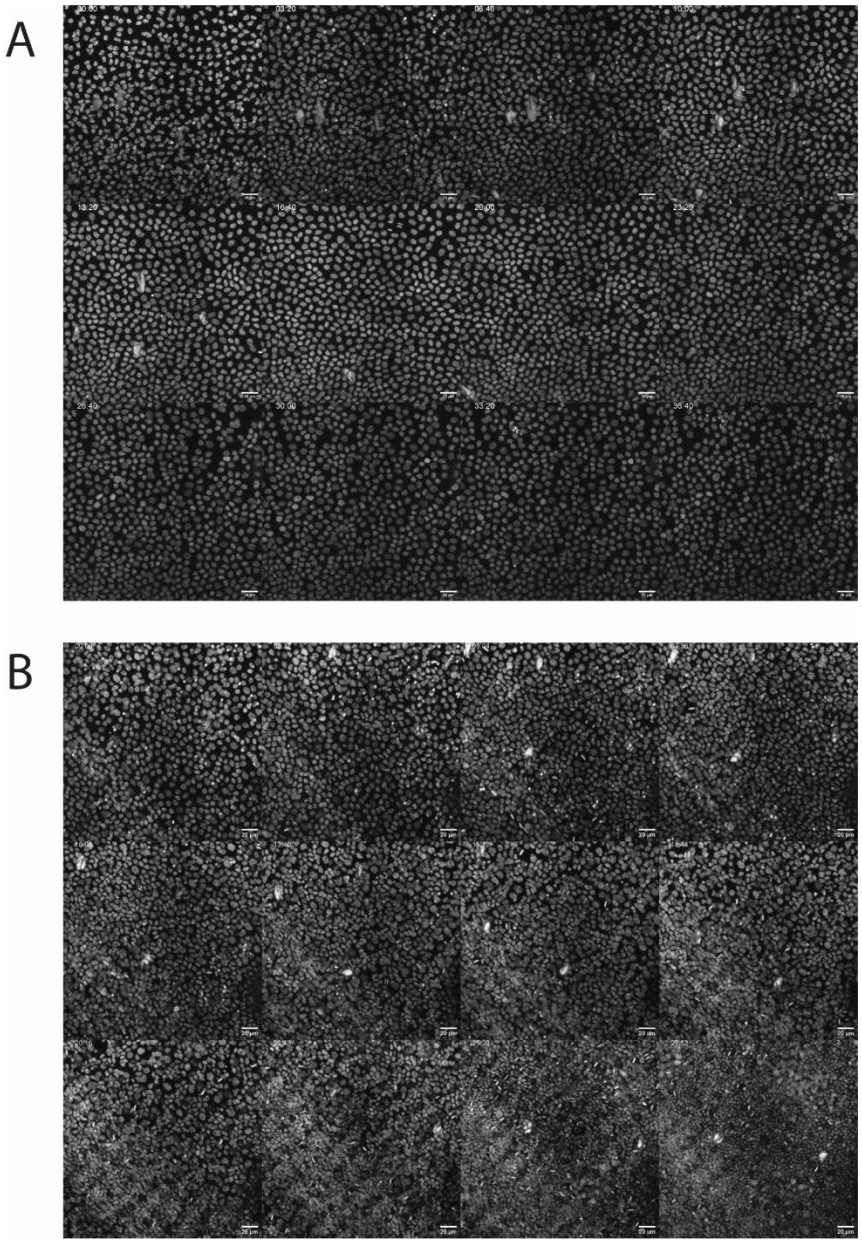
Montage of maximum intensity projections of *Plutella xylostella* wing nuclei labeled with Hoechst33342 from 30min after pupation (designated '00:00') to scale cell endoreplication and elongation (27:52). Montage created from sampling 02:32 intervals from a movie where acquisitions were generated every 8min. Scale bars measure 20µm. Times recorded in hr:min.

Sup. I.4 Two-photon imaging of *Trichoplusia ni* nuclear divisions



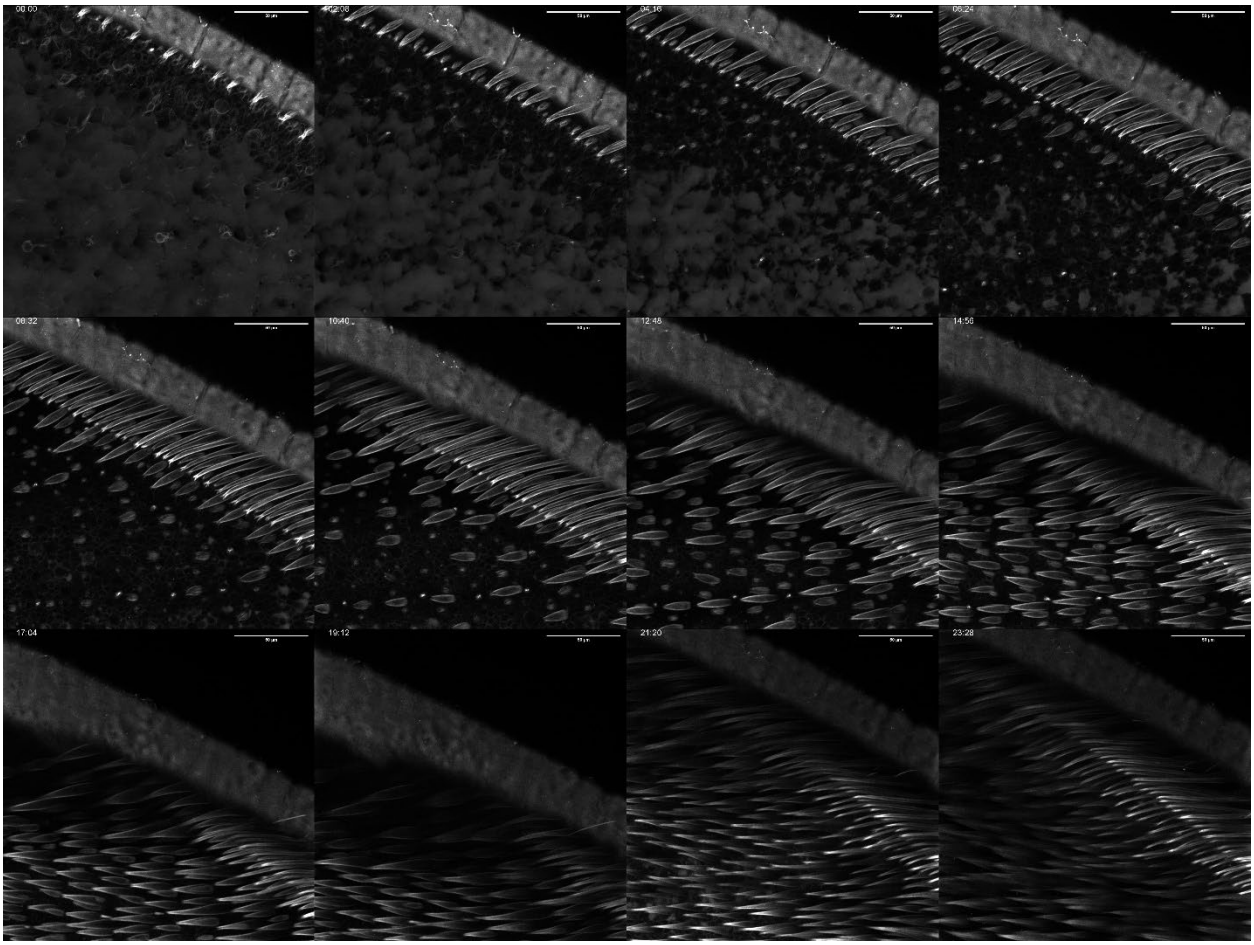
Montage of maximum intensity projections of *Trichoplusia* wing nuclei labeled with Hoechst33342 from 24hr after pupation (designated '0min') to scale and socket cell division (22hr or 1,320min). Montage created from sampling 2hr intervals from a movie where acquisitions were generated every 8min. Scale bars measure 10µm.

Sup. I.5 Two-photon imaging of *Junonia coenia* nuclear divisions



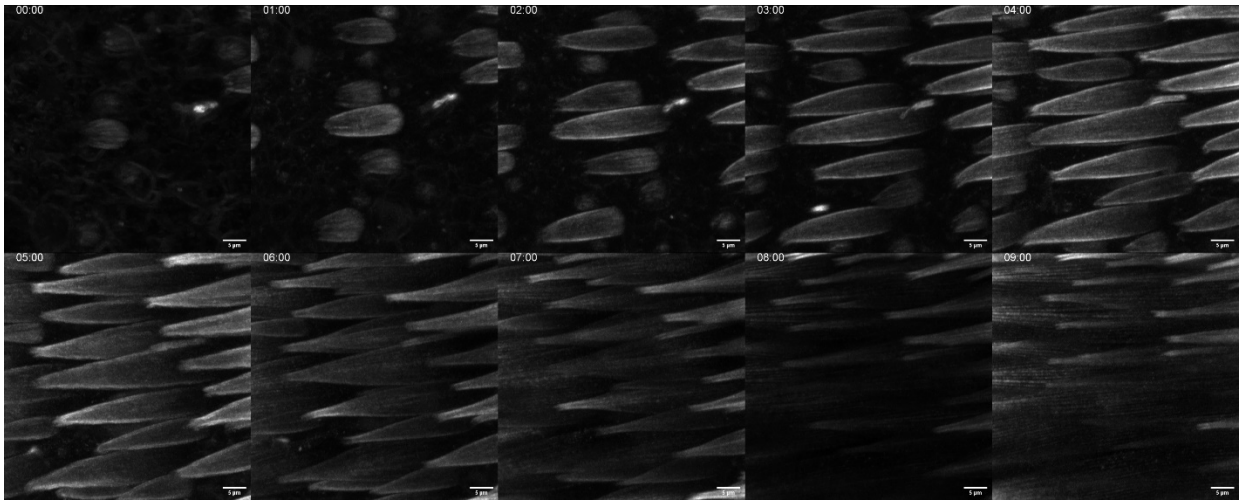
Montages of maximum intensity projections of *Junonia coenia* wing nuclei from two individuals labeled with Hoechst33342 from: A) 1hr after pupation ('00:00') to 36:40 after pupation and B) 36hrs after pupation ('00:00') to 66hrs after pupation ('30:00'). Montage created from sampling A) 03:20 and B) 02:32 intervals from movies where acquisitions were generated every 8min. Scale bars measure 20μm. Times recorded in hr:min.

Fig. I.3 Comparative actin organization in *Plutella xylostella* wing edge scales



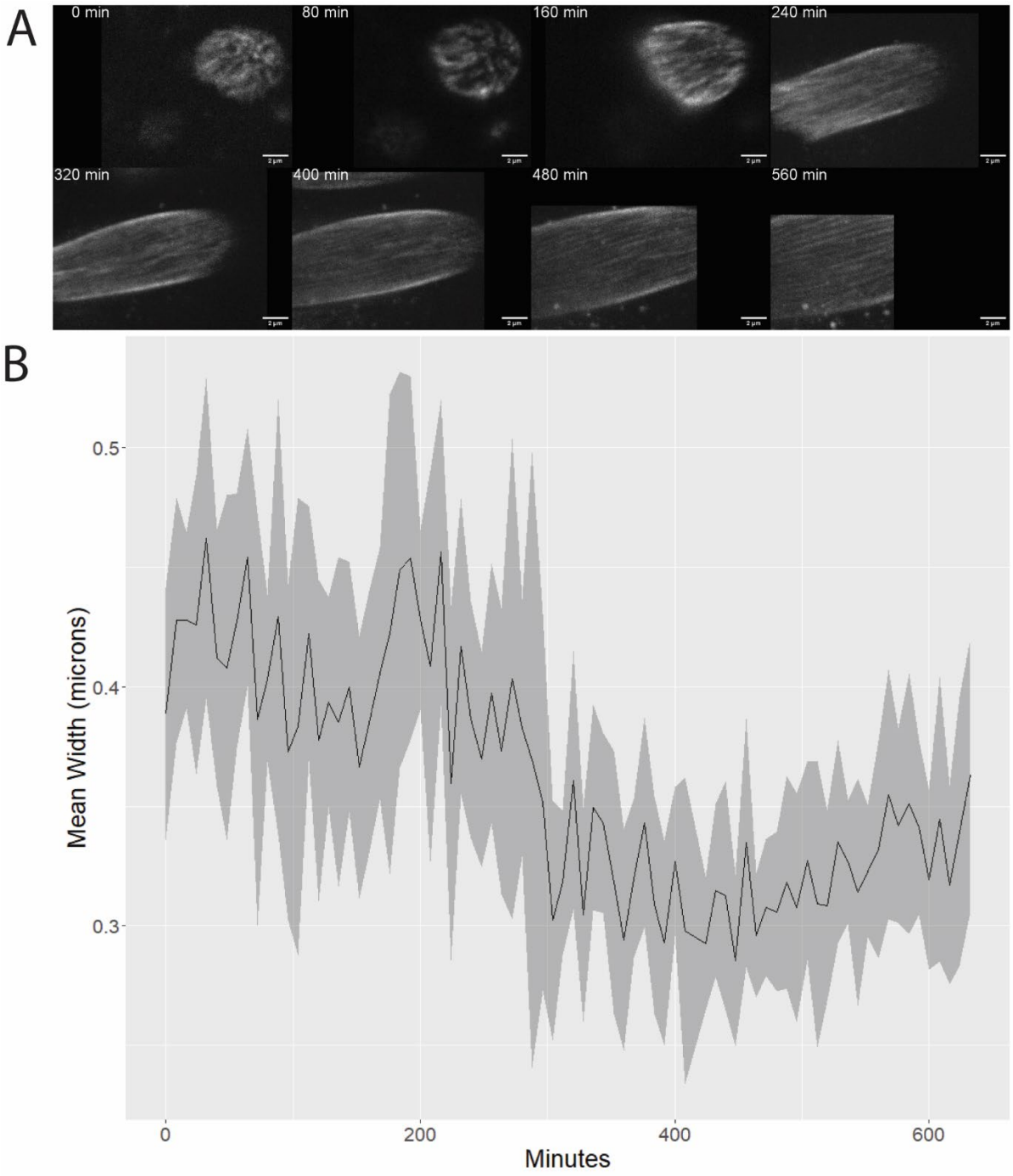
Maximum intensity projection of *Plutella xylostella* scale growth along the wing edge from approximately 24hrs after pupation ('00:00') to 48hrs after pupation with scales labeled for filamentous actin with SiR-Act. Montage frames were sampled at 2:08 intervals from a movie where images were acquired every 8min. Scale bars measure 50µm. Times recorded in hr:min. Scale distal ends are pointed toward the right of the frame.

Fig. I.4 Early actin organization in growing scales



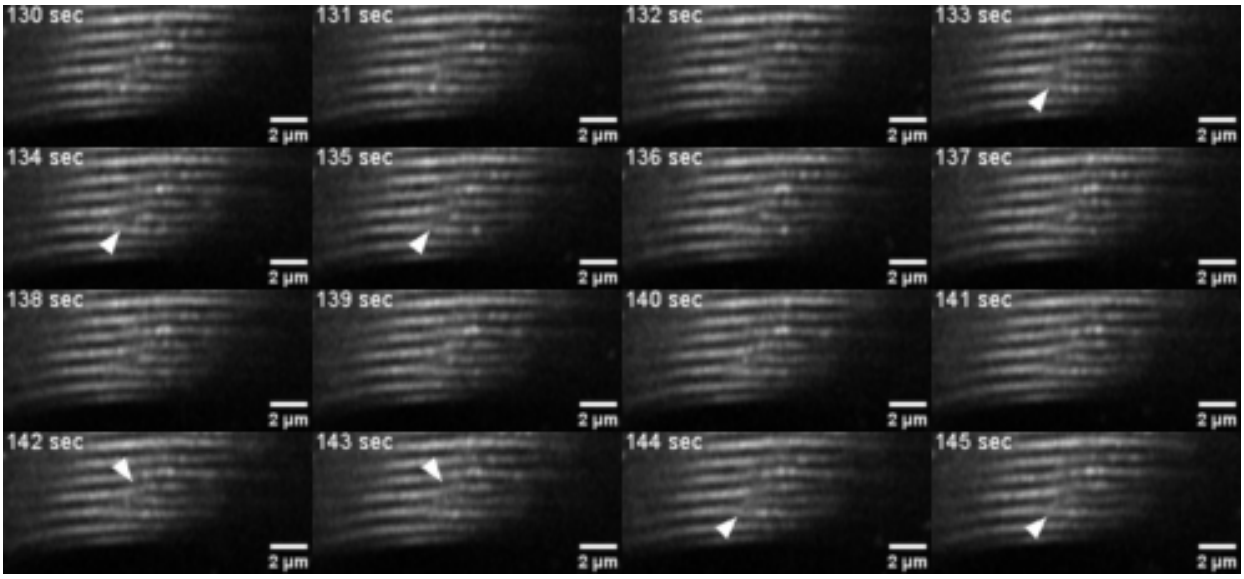
Maximum intensity projection of *Plutella xylostella* scale growth in the wing interior from approximately 24hrs after pupation ('00:00') to 33hrs after pupation with scales labeled for filamentous actin with SiR-Act. Montage frames were sampled at 1:00 intervals from a movie where images were acquired every 3min. Scale bars measure 5µm. Times recorded in hr:min. Scale distal ends are pointed toward the right of the frame.

Fig. I.5 STED imaging of early scale growth suggests phases of actin bundling



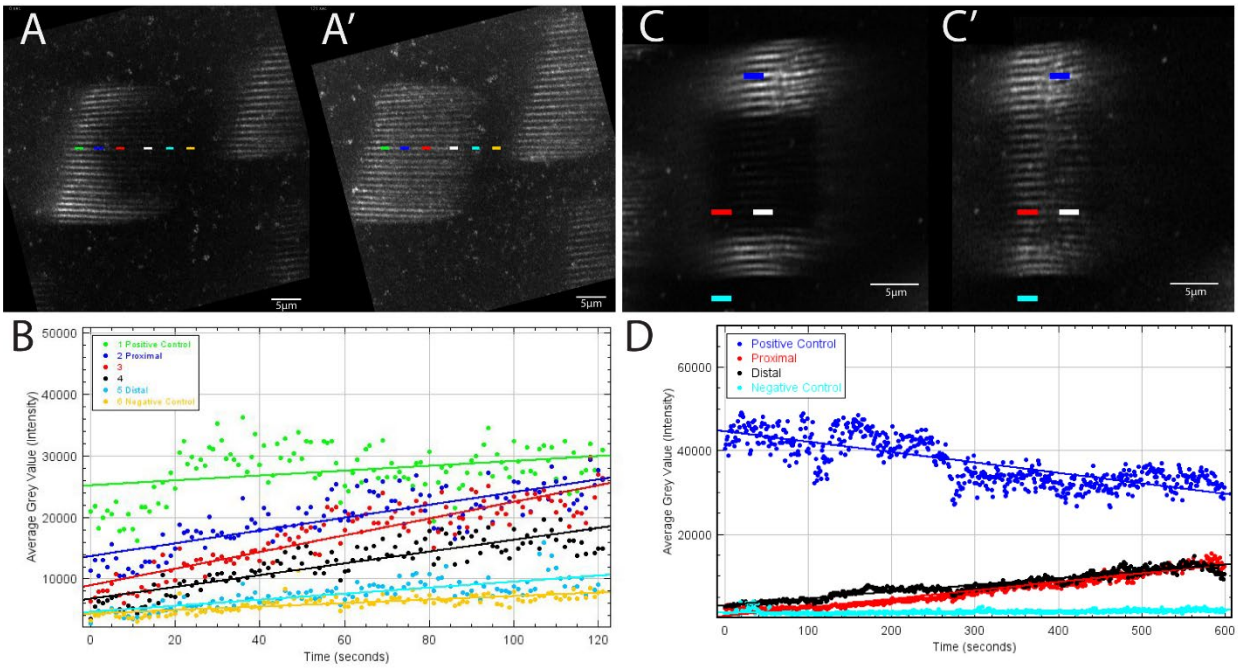
A - Maximum intensity projection of *Plutella xylostella* scales in the wing interior labeled for filamentous actin with SiR-Actin (A). Approximately 24hrs after pupation is designated as '0min'. Montage frames were sampled at 80min intervals from a movie where images were acquired every 8min. Scale bars measure 2µm. **B -** Mean width of actin 'rod' subsets vs developmental time measured from (A), with light grey ribbon representing one standard deviation. Samples sizes for each timepoint plotted in App.I.1. The distal end of the scale is pointed toward the left of the frame.

Fig. I.6 Actin rod dynamics in late scale growth explain aberrant forked scale ridges



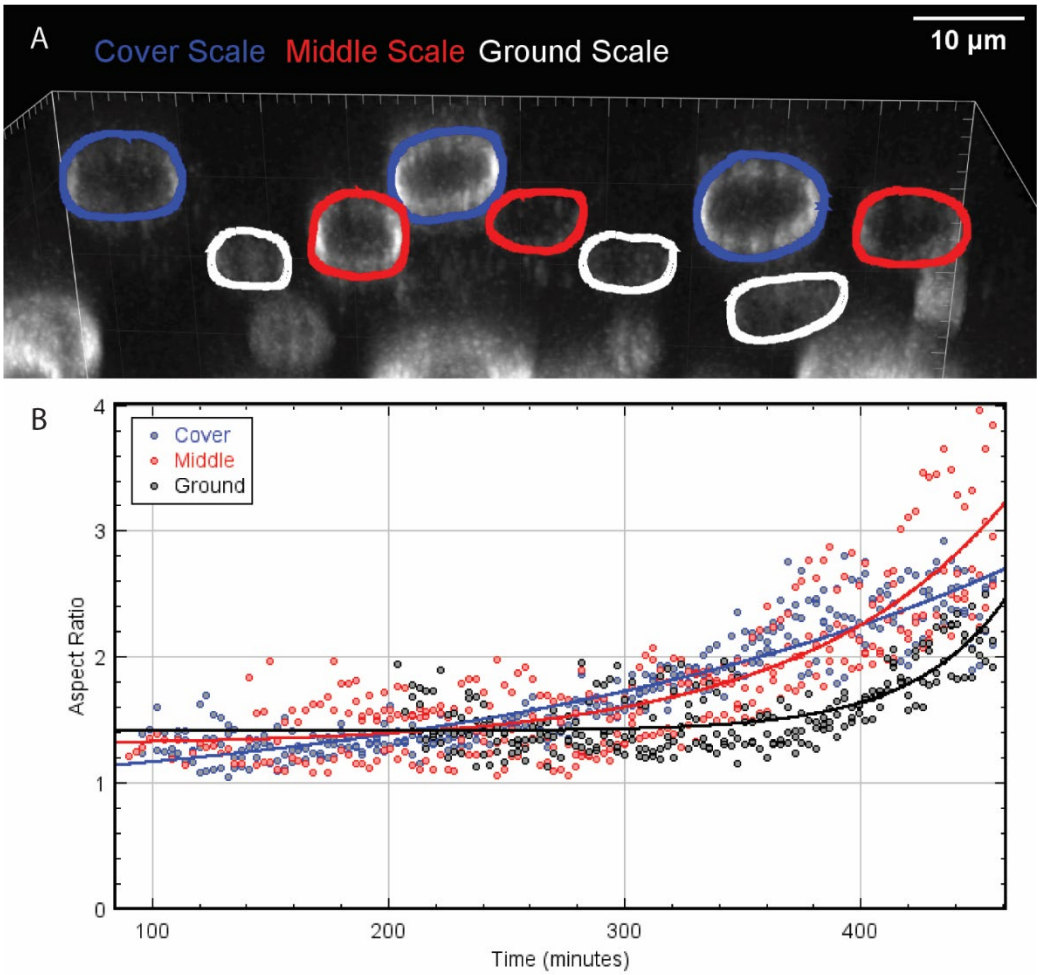
Plutella xylostella actin 'rods' along the lateral edge of a wing interior scale labeled with SiR-Act sampled from an approximately 48hr-old pupa. Montage images represent 15sec extracted from a 200sec long movie where images were acquired as single slices every 1sec. White arrowheads indicate contact sites between a focal aberrant, interleaving actin 'rod' and its neighbors. Scale bars represent 2µm. The scale distal end is pointed toward the right of the frame.

Fig. I.7 FRAP demonstrates actin rod filaments grow distally and then bundle interiorly



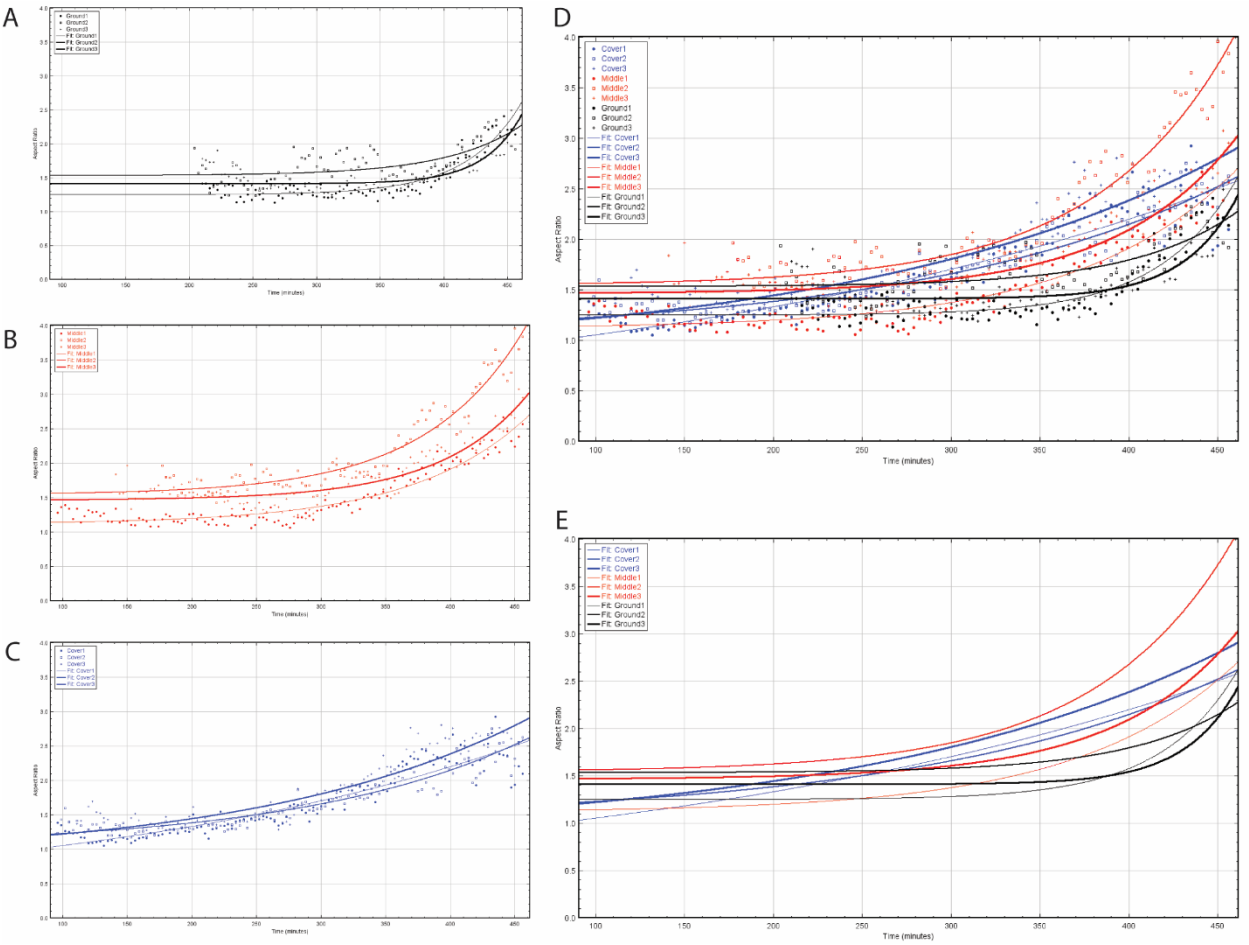
Wing interior scales from ~48-hour old *Plutella xylostella* pupae immediately after photobleaching a square section at the distal tip (A) or scale center (C) and after fluorescence recovery (A', C'). Colored boxes correspond to single-slice ROIs sampled along a posterior-to-distal gradient down an actin 'rod' (lower numbers = more proximal), outside the bleached area ('Positive Control'), and off the scale ('Negative Control') to create FRAP curves of the corresponding color (B, D) (black points substituted for white boxes in A&C panels to maintain contrast in the visualization). Equations for lines of best fit available in Appendix.I.2. and Appendix.I.3. Scale bars represent 5µm. Scale distal ends are pointing to the right of the frame.

Fig. I.8 Change in aspect ratio across developmental time in three moth scale classes



A- An xz projection of Fig.I.4 showing stratification of three scale types in *Plutella xylostella* wings: cover (blue), middle (red), and ground (white) as they elongate towards the viewer. **B-** Aspect ratio vs time plot of measurements from three scales each of the three classes with coloring as in (A) except white is swapped for black. Lines of best fit are imposed exponential growth with delay curves and equations can be found in Appendix.I.4. Measurements obtained starting from ~26hours after pupation. Scale bar measures 10µm.

Sup. I.6 Individual scale heterogeneity in aspect ratio across classes



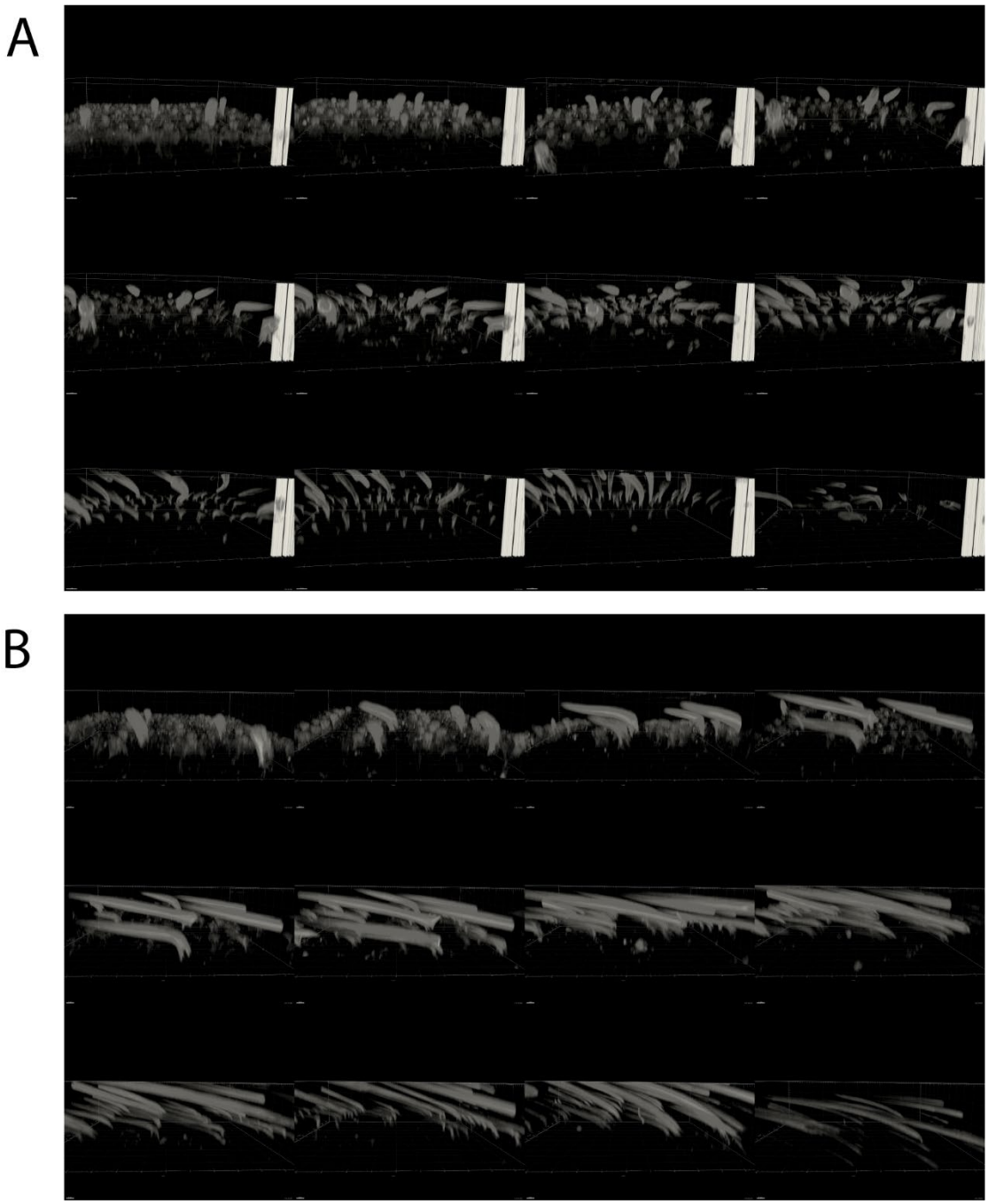
Aspect ratio vs developmental time plots for individual *Plutella xylostella* ground scales (A, black), middle scales (B, red), and cover scales (C, blue) used to create the averages in Fig.I.8 and ultimately measured from an xz projection of Fig.I.4 starting at ~26hr after pupation. Individual scale measurements are indicated with closed circle, open circle, and plus sign marks. Lines of best fit are imposed exponential growth curves with offset and distinguished for each measurement by thickness (closed circle = thinnest line, open circle = intermediate line, plus sign = thickest line). Equations for curves are available in AppendixI.5. All measurement points and imposed curves of best fit for each scale class, with colors, symbols, and thicknesses maintained from (A), (B), and (C), are plotted together in (D) and the imposed curves isolated in (E).

Fig. I.9 Comparative actin organization in butterfly cilia and interior wing scales



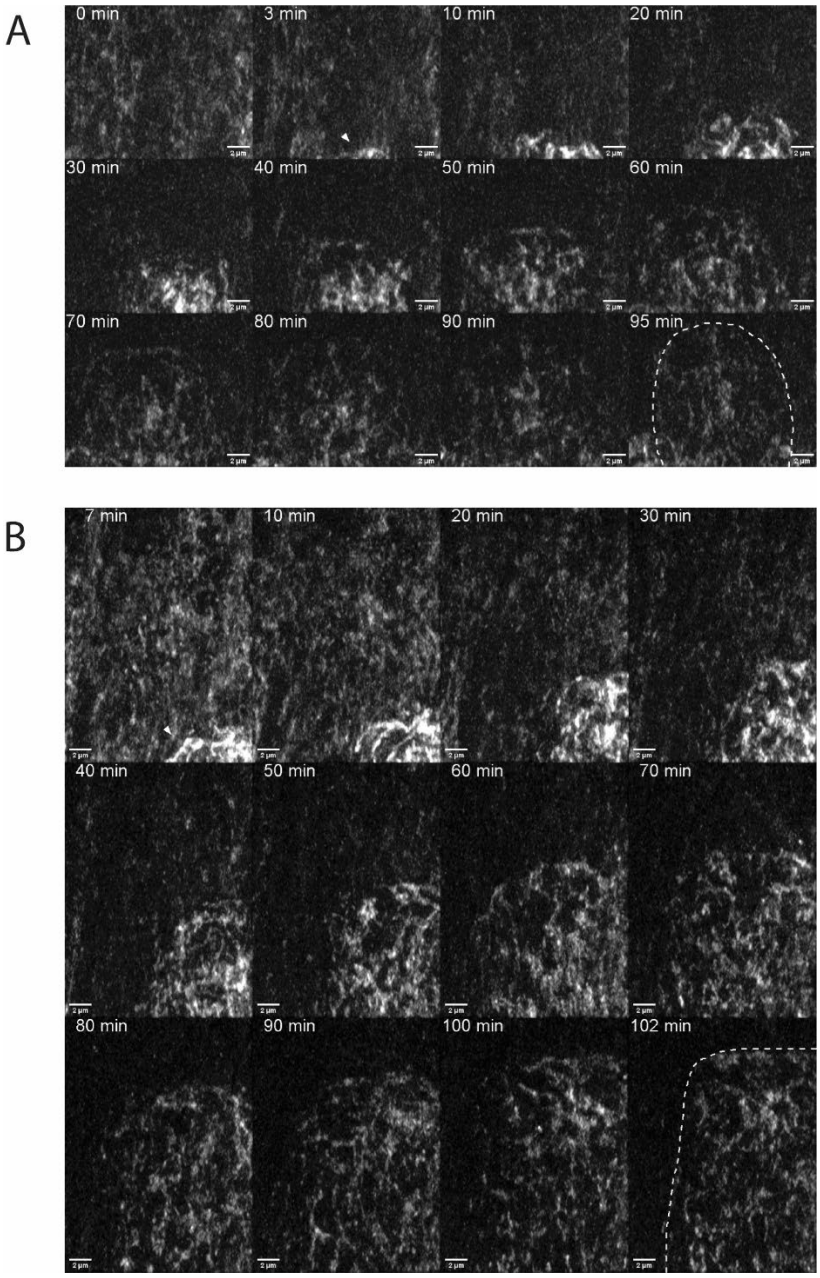
Junonia coenia scale growth maximum intensity projection in the proximal wing from approximately 72hrs after pupation ('00:00') to ~100hrs after pupation with scales labeled for filamentous actin with SiR-Act. Montage frames were sampled at 184min intervals from a movie where images were acquired every 8min. Scale bars measure 10μm. Scale distal ends are pointed to the top of the frame.

Sup. I.7 Cilia scales have a cylindrical growth onset



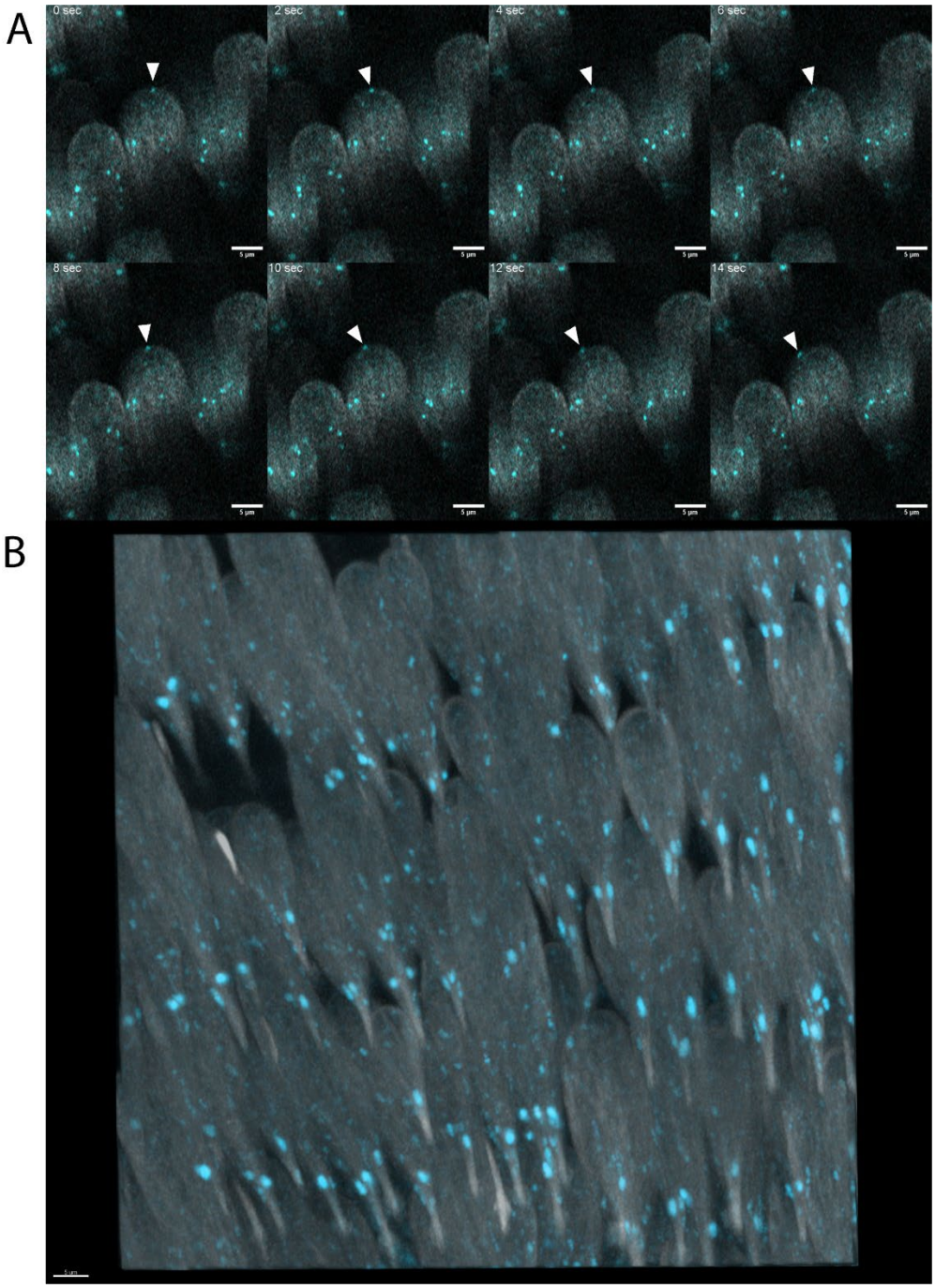
Junonia coenia scale growth in the proximal wing from Fig.I.9 in xz "extending-toward-viewer" termed "head-on" (A) and yz "profile" (B) projections from approximately 72hrs after pupation ('00:00') to ~100hrs after pupation with scales labeled for filamentous actin with SiR-Act. Montage frames were sampled at 140min intervals from a movie where images were acquired every 8min. Large tick marks on the bounding frame correspond to 1µm. Scale distal ends are pointed toward the viewer (A) and to the left of the frame (B).

Fig. I.10 Mitochondrial organization in growing distal tips of wing scales



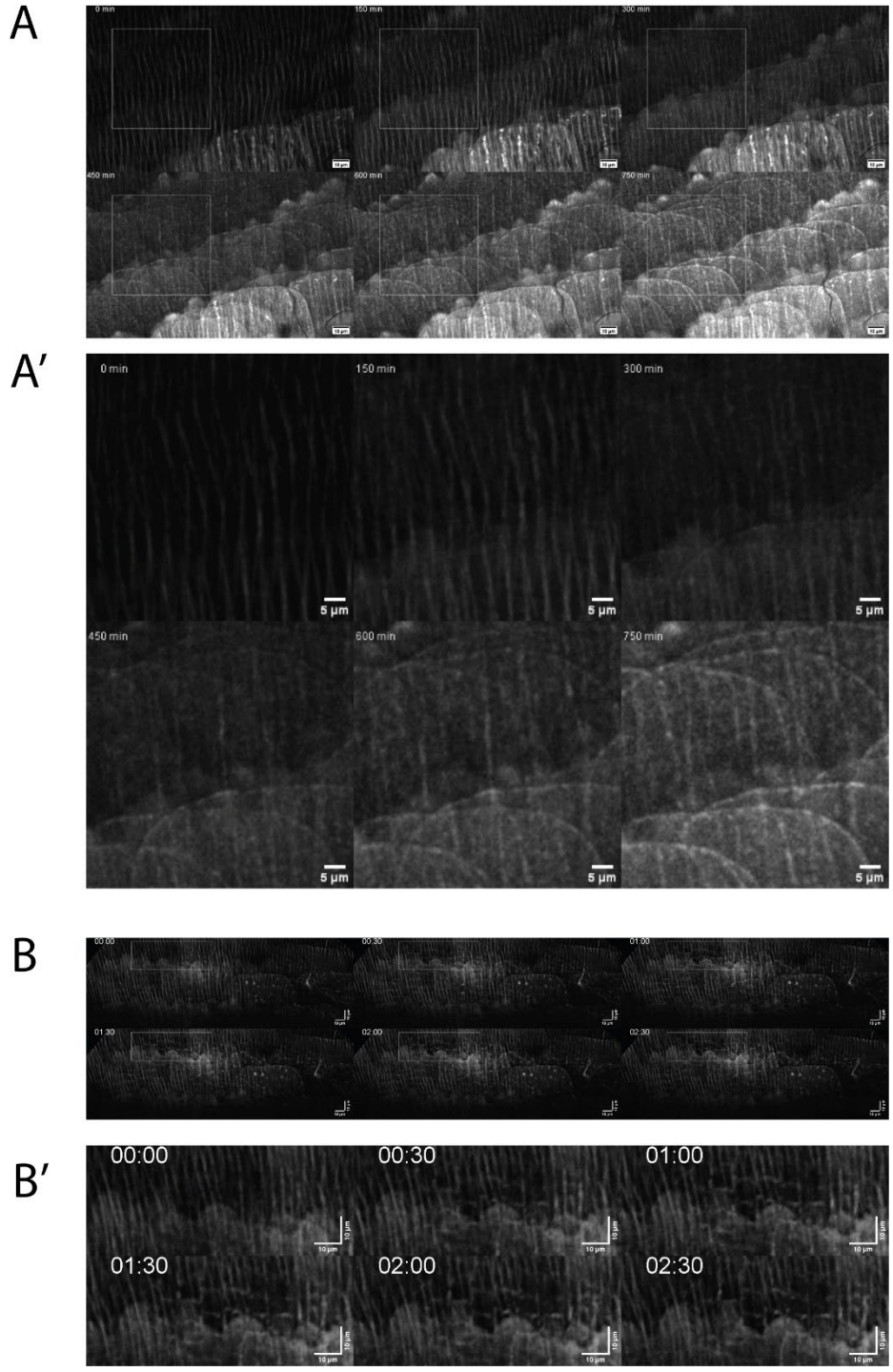
Maximum intensity projections of growing distal tips of two individual *Plutella xylostella* wing interior scales (A, B) from approximately 36hrs after pupation ('0') to ~38hrs after pupation with scales labeled for mitochondria with MitoTrackerRed. Montage frames were sampled at 3min intervals from movies where images were acquired every 1min. White arrow heads in the first frames point to the growing distal scale tip, whereas dashed lines in the final frames approximate scale cell borders. Scale bars measure 2µm. Scale distal ends are pointed to the top of the frame.

Fig. I.11 Vesicle transport and distribution along scale microtubule networks



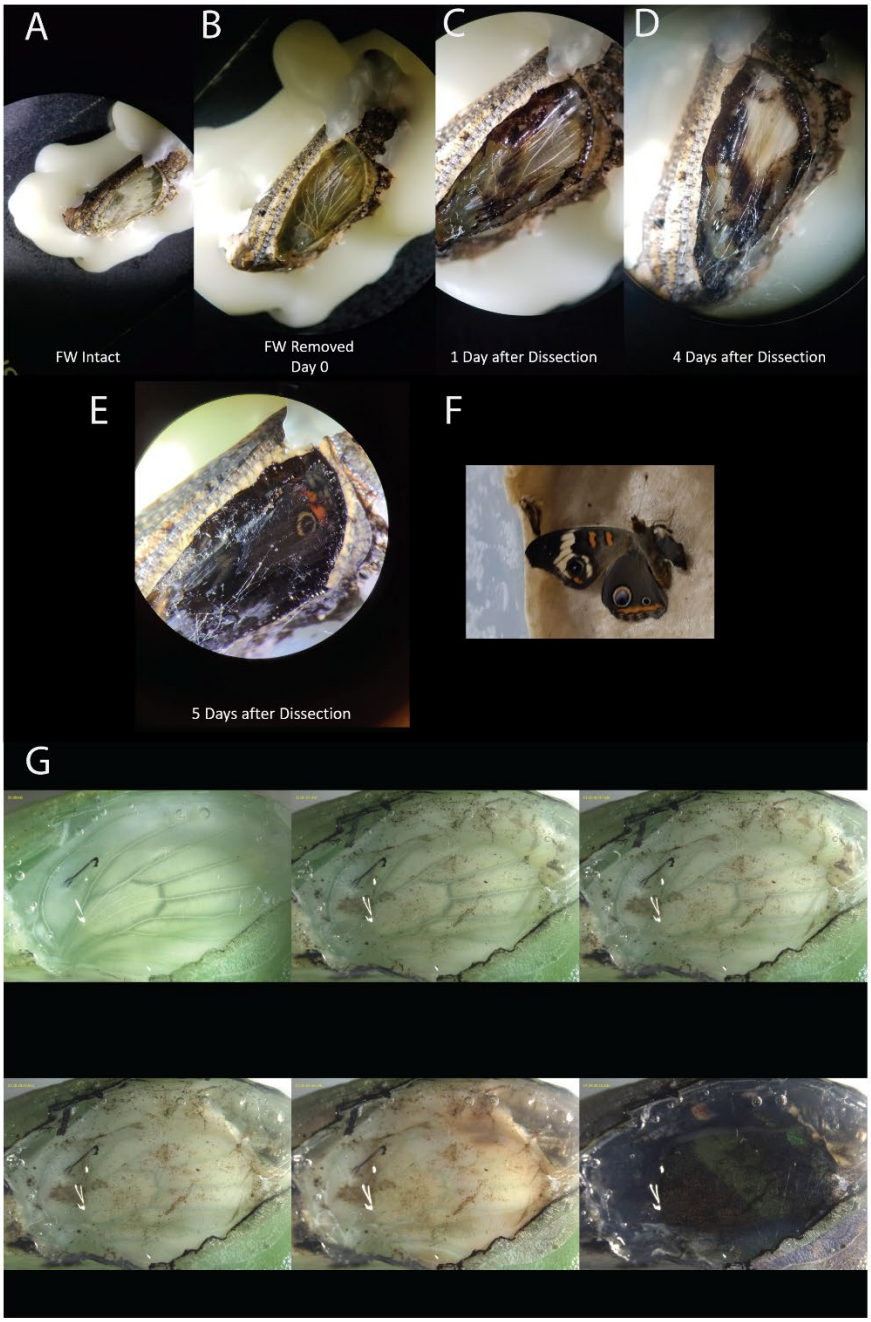
Plutella xylostella wing interior scales at approximately 48hrs after pupation labeled for microtubules (grey) and lysosomes (cyan) using TubulinTracker Green and LysoTracker Deep Red. **A-** Montage frames represent a 14sec excerpt of 1sec interval single slice acquisitions from a 1min long movie with white arrowheads tracking the position of a single vesicle along a microtubule. **B-** Maximum intensity projection of scales from (A). Scale bars measure 5µm. Scale distal ends are pointed to the top of the frame.

Fig. I.12 Two-phase actin restructuring in structurally green swallowtail scales



Papilio palinurus structurally colored green wing band scale maximum intensity projection (A) and single frame (B) montages starting from an intermediate scale growth stage to ~12hr after, with scales labeled for filamentous actin with SiR-Act. Montage frames were sampled at 150min (A & A') and 30min (B & B') intervals from movies where images were acquired every 15min. (A') and (B') are magnified views of the white boxed region outlined in (A) and (B). Scale bars measure 10µm (A, B, & B') and 5µm (A'). Scale distal ends are pointed to the top of the frame.

Sup. I.8 Live forewing dissection photographic examples



A *Junonia coenia* pupa (A) immediately after removal of the forewing cuticle, (B) immediately after vivisection of the forewing, (C) the day after forewing removal, (D) four days after forewing removal, and (E) five days after forewing removal with normal scale growth/wing pigmentation, and (F) eclosed six days after forewing removal (mounting in VALAP and lack of forewing support lends the hindwing to becoming crumpled). **G-** Forewing-dissected *Papilio palinurus* pupal montage taken from an ~4.5day timelapse imaged in 2hr intervals. Montage frames sampled every 00:22:30:00. Times recorded in days:hours:minutes:seconds. Wings oriented with distal pointing toward the right of the frame.

Chapter II: Cytoskeletal and Genetic Contributors to Cylindrical Scale Shapes in Lepidoptera

II.1 Introduction

Lepidopteran scales span a continuous variation that has principally been subdivided according to outline, cross-section, and presence of microstructures (Kellogg 1894; Downey & Allyn 1975). Amazingly, scale shape diversity arises from differences in the ultimate form of individual giant trichogen cells in the pupa (Nijhout 1991). These cells lay below the epithelial sheet and extend extremely large apical processes which form the approximate outline of the scale as they reach their apex in growth. At this point, they change their membrane geometry in defined phases to adopt the shape of sequential layers of the adult structure as they shrink inwards, secreting cuticle along the way—akin to a 3-D printer working out-to-in. At the end of this secretion process, the adult lepidopteran emerges from the pupa covered in the sclerotized, chitinous scales, effectively the husks of the trichogens before them (Stossberg 1938).

Trichogens themselves are derived from sensory organ precursor cells which have diversified in lineages throughout arthropods to produce the gamut of epithelial projections from setae, to bristles, to even other convergent types of scales (Lai & Orgogozo 2004; Westphal-Ferreira & Navarro-Silva 2017). These anatomical classes, like ultimately most, are artificial categories splitting continuous variation according to the presence or absence of certain characters deemed most representative. Generally, these macrochaete designations fall between two extremes: setae/bristles, which are piliform, cylindrical in transverse section, and have circumferential ridges, and scales, which are lamelliform, ovular in cross section as a result of flattening, and have ridges on only one surface (Gordh & Headrick 2011). Yet, many cases of macrochaetae which fall into intermediate designations exist, like being only partially flattened, being flattened with ridges on each side, or being cylindrical proximally and flattened apically, among a seemingly infinite array of combinations. Historically, in the Lepidoptera, every non-innervated macrochaete is referred to as a type of scale, perhaps in recognition of the arbitrary nature of the distinction, and this work follows this tradition while maintaining respect for their homology to the other classes (Kristensen & Simonsen 2010; Galant *et al*. 1998; Zhou *et al*. 2009).

Scales of different colors are shown to share similar microstructural elements (Janssen *et al*. 2001; Gilbert *et al*. 1988; Aymone *et al*. 2013). Furthermore, genes specifying or influencing scale color have been extensively characterized in a fruitful branch of lepidopteran genetics (Beldade & Brakefield 2002). Different classes of colored scales in different species have been found to be defined by the expression of the same "master regulator" transcription factors. This relationship is best understood in *Heliconius melpomene*, where, in addition to roles in wing patterns broadly, red scales express *optix*, *aristalless1* determines white over yellow scales, and *cortex* seems to regulate the split between white/yellow and red/black, specifying the latter (Reed *et al*. 2011; Westerman *et al*. 2018; Livraghi *et al*. 2021). Transcription factors determining structurally colored scale types, too, have been identified. *Antp* and *optix* were found to differentiate a silver scale identity in *Bicyclus anynana* (Prakash *et al*. 2022). In *Junonia coenia*,

optix, again, specified a blue structural color while its direct target, paired with *spalt*, *araucan*, was implicated in having a role in determining its hue (Zhang *et al*. 2017; Chatterjee *et al*. 2023 preprint). Outside the human visual spectrum, *Colias eurytheme* non UV-iridescent scales express *bric a brac*, indicating a role in repressing the formation of precisely stacked lamellae on abwing scale ridges (Ficarrotta *et al*. 2022). Other genes determining the production and transport of pigments can have effects on wing scale ultrastructure, likely through interfering with properties of the cell membrane and cuticle and interfering with stereotyped physical processes in scale development, like the opening of windows into the lumen upon exposure to air after eclosion (Matsuoka & Monteiro 2018).

Yet, at its core, scale variation arises due to changes in the downstream mechanisms dictating shape changes in the apical processes of trichogens as they grow. Changes in cell morphology are largely driven by restructuring in the organization of cytoskeletal polymers and this has already been demonstrated to be true in the case of lepidopteran scales (Salbreux *et al*. 2012; Tilney & Porter 1967; Tilney & Gibbons 1969). Abwing ridges are prefigured as the cells elongate by parallel fibers of highly bundled filamentous actin—termed 'rods'—that stretch from the proximal to distal ends (Dinwiddie *et al*. 2014). Once scales have reached their total length, cortical actin reorganizes to form microribs, lamellae, and trabeculae (Lloyd *et al*. 2023 preprint; Seah & Saranathan 2023). Continuous live imaging has revealed the dynamics of the actin cytoskeleton in these instances, confirming that scales elongate from their distal tips like homologous *Drosophila* bristles, with polymerization apically and bundling interiorly (Chapter I). Furthermore, it has shown in combination with serial dissections, that the typical reorganization of late-stage cortical actin can be commandeered in the evolution of structural coloration to produce elements which constructively and destructively interfere with visible wavelengths of light (Null 2017). Microtubules have been less well characterized in scale growth, partly due to them being long discounted as being minimally dynamic and having a much lesser role than actin in *Drosophila* bristle elongation (Tilney *et al* 2000). Nonetheless, transmission electron micrographs have shown changes in their distribution throughout development and a particularly structured, novel organization in the bristle-like cylindrical scales of the glasswing *Greta oto* where they are positioned interior and parallel to the actin filaments (Overton 1966; Pomerantz 2021). These findings heavily imply that microtubules may play a larger, as yet uncharacterized, role in scale morphogenesis than in that of bristles.

Scales are heterogeneous in both shape and color, with this variation not being mutually exclusive. That is to say, scale types of the same color, and thus general microstructure and pigment distribution, can have wildly different forms. While the genetic control of microstructural architecture as it relates to types of color identity is reasonably well understood, as outlined above, considerably less is understood about how the differences in scale outline manifest. A particular axis of variation that has been repeatedly identified in the literature is how scales along the wing edge are much longer than those in the interior (Dohrmann & Nijhout 1989). At the same time, there are scale types within the wing interior that more closely resemble these marginal scales and are cylindrical and much longer than their flattened neighbors (Pan *et al*. 2022). These piliform scales or 'cilia' are fairly common throughout Lepidoptera and ascribed

to pheromone-dispersing and thermoregulatory functions; but, taken to the extreme, some species have evolved extensive coverings to expose colored patches of wing membrane or, in the "glasswing" butterflies, an almost complete covering of bristle-like scales as an ostensible form of camouflage that "matches" every background (Kristensen & Simonsen 2001; Pivnick *et al*. 1992; Heinrich 1987; Gomez *et al*. 2021). This class of scales presents an interesting avenue to pursue factors influencing overall form. Namely, it may be possible to identify factors which are expressed along the wing edge that have been evolutionarily recruited to shift interior scales into a more marginal-type phenotype. The gene *cut* is a particularly interesting candidate in this respect, as it is 1) expressed along the margin and outside the wing and determines the adult wing shape early on by shunting non-wing lacuna cells to an apoptotic fate, 2) characterized to specify between chordotonal organ and bristle macrochaete classes from *Drosophila* mutagenesis, and 3) is known for being continually expressed in sensory organ precursor cells and their daughters throughout growth, suggesting that its continued presence is necessary to maintain a specific growth regime (MacDonald *et al*. 2010; Bodmer *et al*. 1987; Blochlinger *et al*. 1988; Blochlinger 1990).

Altogether, there is a gap in our understanding about how the different cytoskeletal polymers may interact in the context of different scale morphotypes across ontogeny and phylogeny and which genetic factors might be specifying different forms of cell elongation and growth. This study compares the organization of actin and microtubule cytoskeletons with localization of the Cut protein to understand the relationship between cellular organization and gene regulation of the piliform-lamelliform morphological axis across a range of Papilionoidea with cylindrical scales morphologies. *Greta oto* and *Methona confusa* are two distantly related ithomiine nymphalids with modified bristle-like scales covering their wings (Willmott & Freitas 2006). *Junonia coenia* is a junoniine nymphalid with androconial cilia along the interior border of the hindwing vannal fold. *Graphium agamemnon* is a papilionid with modified colorful membrane patches on the fore- and hindwings that are covered in piliform scales of two types: peg-like scales on the dorsal side and cilia-like scales on the ventral side (Allyn *et al*. 1982). And *Plutella xylostella* diamondback moths are used as an outgroup and to test the generalities of the cytoskeletal associations recorded amongst the butterflies with empirical manipulations, as they do not have cylindrical scales outside the sensory scales along the veins and wing margins. Additionally, in a first step to determine downstream factors of Cut transcription factor activity, a method is devised to dissociate pupal wing tissue for single cell RNA profiling of individual scale transcription.

II.2 Methods

II.2.1 Animal husbandry

Plutella xylostella strain G88 were maintained in staggered colonies at 25ºC or 27ºC and natural or 16:8 (day:night) light cycle, respectively, and larvae fed on wheat germ-based diamondback moth artificial diet supplemented with linseed oil (Southland Products Incorporated) in Styrofoam cups. Adults were fed on a 12% sucrose solution dyed with yellow food coloring.

Junonia coenia from a lab-adapted colony were gifted by Fred Nijhout (Duke University) and maintained on multiple species diet (Southland Products Incorporated) mixed with powder made from dried *P. lanceolata* leaves using the following ratio: 4.4g *Plantago*:61.6g artificial diet powder in 350mL H_2O. Adults were fed Gatorade (red flavor) soaked into kitchen sponges.

Methona confusa and *Graphium agamemnon* pupae were reared in Ecuador and the Philippines, respectively, and purchased through London Pupal Supply, LLC. *Greta oto* from a resident colony were purchased from Magic Wings Butterfly Conservatory & Gardens (281 Greenfield Rd, South Deerfield, MA 01373). *M. confusa* and *Greta oto* were kept under USDA Permit P526P-22-07598 and housed in approved facility 5046. *Graphium agamemnon* were shipped to and dissected at Magic Wings under their permit.

II.2.2 Immunohistochemical staining

Pupal wing tissues of desired developmental stages were dissected in 4°C PIPES:EGTA:$MgSO_4$ (PEM) buffer and fixed in 3.2% paraformaldehyde in PEM for 30min at room temperature (Cold Spring Harbor Protocols 2009). Fixed tissues were washed 3x5min and 2x30min in 1mL of phosphate-buffered saline (PBS) before adding 300µL of 1:200 wheat germ agglutinin (WGA) conjugated to Alexa Fluor 488 (Invitrogen, W11261) in PBS for 1hr at room temperature to stain N-acetylglycosamine (GlcNAc) residues in the cell membranes (hereafter 'WGA') (Monsigney *et al*. 1979). Tissues were washed 3x5min, then 3x5min and 3x30min in PBS with 0.1% Triton X-100 detergent (PT) to permeabilize the tissue before blocking with 300µL of 5% normal donkey serum (NDS) in PT for 30min at room temperature.

Blocked wings were split into two forewing-hindwing pairs. The first pair were stained with 1:10 mouse α-Cut monoclonal primary antibody (DSHB, 2B10) to target Cut protein localization (Blochlinger *et al*. 1990). These wings were incubated overnight at 4°C, washed 3x5min then 3x30min in PT. Again, wings were blocked in 5% NDS in PT for 30min at room temperature followed by the addition of 1:300 donkey α-mouse IgG secondary antibody conjugated to Alexa Fluor 594 (Invitrogen, A-21203). Wings were kept in the secondary for 2hrs then washed 3x5min and 3x30min in PT. In a subset of *J. coenia*, wings, 1:500 goat α-horseradish peroxidase conjugated to Alexa Fluor 488 (Jackson ImmunoResearch, 123-545-021) was added in the primary antibody solution to label neuron surfaces (hereafter 'HRP') (Jan & Jan 1982).

The second pair of wings were stained with 1:200 Alexa Fluor 555-conjugated phalloidin (Invitrogen, A34055) and 1:500 mouse α-acetylated α-tubulin monoclonal primary antibody (6-11 B-1) conjugated to Alexa Fluor 647 (Santa Cruz Biotechnology, sc23950 AF647) to label the filamentous actin and acetylated α-tubulin, respectively (Wulf *et al*. 1979; LeDizet & Piperno 1991). Wings were incubated overnight at 4°C then washed 3x5min and 3x30min in PT.

1µL 1µg/mL DAPI to label DNA was sometimes added in the primary antibody staining solutions to save time (Williamson & Fennell 1975). Otherwise, after the final wash step, both sets of wings were stained in 1µg/mL DAPI in 50% glycerol (DAPI-glycerol) for 2hrs at room temperature, then stored in 70% glycerol at 4°C.

II.2.3 ACME fixed tissue single cell dissociation and sequencing

Pupal wings of desired stages were dissected in ice-cold Grace's insect media (Gibco) and processed using a modified ACME (ACetic MEthanol) dissociation protocol (García-Castro *et al*. 2021; Appendix II.1). Briefly, this protocol differs in 1) defining optimal periods of regular mechanical disruption of the tissue in the ACME solution throughout shaking to completely separate cells and 2) filtering of suspensions from late-stage wings to remove debris and elongated scale projections that break off at the pedicel. Samples were prepared into GEMs using 10X Genomics Chromium controller. 3' scRNAseq libraries were sequenced on an Illumina NovaSeq with an SP flowcell with 100bp cassettes at the University of Chicago Genomics Facility. Sequencing summary statistics were made using the 10X Genomics Cloud Analysis tool.

II.2.4 Imaging and microscopy

Individual scales were removed from adult wings using a sharpened tungsten needle and arranged on glass slides. Color micrographs of these series were taken using a Keyence VHX7000 microscope using reflected light and the glass plate stage insert. Scales mounted similarly on carbon tape were sputter coated with ~3nm platinum and scanning electron micrographs taken with a ZEISS Supra 40VP using the secondary electron (SE2) detector. The electron high tension (EHT) was set at 3.0kV at a working distance of 13.2mm.

Stained wings were mounted using supports made from #1 and #1.5 coverslips in 70% glycerol on glass microscope slides. Images were acquired with a ZEISS LSM880 Airyscan inverted confocal with the following ZEISS objectives: 5x Fluar 0.25NA, Plan-Apochromat 10X 0.45NA, Plan-Apochromat 20X 0.8NA, C-Apochromat 40X 1.2NA water. For protein localization stains, a PMT detector with default filter sets for appropriate Alexa Fluor dyes was used. For each channel, a pinhole of 1Au (Airy unit) was selected. For imaging the cytoskeleton, the Airyscan detector was used. Detection wavelengths in the PMT detector were shifted to avoid overlap in emission curves and all channels imaged individually to avoid bleed-through. Single cell dissociations were imaged on a widefield ZEISS Observer Z.1 compound microscope with Hamamatsu Camera Controller C10600 Orca R^2 and 10X, 20X, 40X, and 100X objectives. An X-Cite Series 120 was used for fluorescence illumination with a Cy3 filter set.

Live imaging of the microtubule cytoskeleton was conducted as described in Chapter I. Briefly, pupae were paralyzed with 1mM tetrodotoxin citrate (Abcam, ab120055 discontinued) in water and pupal wings injected with a 1 part 1mM SiR-Tubulin:1 part verapamil:3 parts Grace's insect media: 11 parts dimethyl sulfoxide (DMSO) mix using a glass capillary needle and Narishige IM-300 microinjector. Injection and imaging were through the peripodial membrane of wing flipped hindwings in 72HAP *J. coenia* and the forewing cuticle in *P. xylostella* and 48HAP *G. oto*. *P. xylostella* were mounted on a glass slide as in Chapter I and *J. coenia* and *G. oto* were placed into #1.5 27mm Nunc glass bottom dishes. Imaging was performed on the above ZEISS LSM880 using the 40X objective, selecting the AiryscanFast detector and Cy5 filter settings.

Taxol-treated *P. xylostella* were injected through the forewing cuticle with 50μM paclitaxel (Abcam, 33069-62-4) in DMSO after the injection mix, injecting at most two droplets using the same settings and needle size. A subset of injected pupae were dissected and stained as above for

filamentous actin and acetylated α-tubulin 16hrs after the injection to better resolve the morphology of taxol-treated scales.

All fluorescence images were adjusted for contrast and annotated using Fiji (Schindelin 2012). Z-frames of individual channels composing maximum intensity projections had to be trimmed separately to allow simultaneous visualization of scales and nuclei in composites for the assessment of Cut protein localization. Live movies were compiled from maximum intensity or standard deviation projections and processed as in Chapter I, with registration using Correct 3D Drift (Parslow 2014). Figures were compiled using Illustrator (Adobe Creative Cloud).

II.3 Results

II.3.1 Actin and microtubule cytoskeletal networks uncouple during scale flattening

During initial projection and the beginning of first extension, F-actin and acetylated α-tubulin (hereafter simply referred to respectively as 'actin' and 'microtubule') cytoskeletal networks display astonishing overlap, with filaments being almost indistinguishable if not for the proper localization of actin to the cell membranes and microtubules to the spindles of still-dividing epithelial wing membrane cells (Fig.II.1A, overlap in composite indicated as white). Yet, by the flattening phase, the parallel microtubules uncouple from the actin cytoskeleton: the actin remaining as parallel rods running basal to distal and microtubules shifting to a reticulated, diffused network in the distal portion of the cell (Fig.II.1C). In the basal portion, in the pedicel region, microtubules remain parallelized and overlapping with actin. Intriguingly, cylindrical sensory scales along the wing veins share this parallel arrangement of microtubules overlapping with actin. And, in a similar manner, the elongated marginal scales along the wing edge have parallel microtubule networks that overlap with actin for far longer in their development, as indicated by the proportion of the cell projection in which these two polymers are overlapping, and the point when the microtubules uncouple into a reticulated network coincides with the flared distal tip characteristic of these cells (Fig.II.1B,D).

II.3.2 Cut protein localizes to cylindrical scale nuclei across Lepidoptera

Specific wing margin factors could possibly have shifted expression to bestow margin scale-like phenotypes to interior scales, with *cut* being a promising candidate for its aforementioned expression along the wing edge and known role to specify between types of sensory organ cell classes in *Drosophila*. Cut protein was indeed found to localize to cilia scale and socket cell nuclei, which are cylindrically shaped scales present in the interior *J. coenia* hindwing (Fig.II.2). Cut was also confirmed to be present along the pupal lacunal cells that will undergo apoptosis to give the wing its overall shape and in innervated sensory scale cells spaced around the wing margin (Sup.II.1B) (MacDonald *et al*. 2010; Yoshida & Emoto 2010). Because sensory scales are Cut-positive and cylindrical, but are also innervated, it was unclear whether Cut was indicating sensory state or a possible cell shape correlation. Staining wings with HRP to identify neural tissue verified nerve processes along the wing veins and innervation of the sensory scale, but showed no signal in the cilia, confirming that they lack a neural component like most scales on the lepidopteran wing (Sup.II.1, asterisks). Similarly, it was possible that Cut expression was

activating a sex-specific pheromone production pathway as cilia have been thought to serve as conduits to wick these chemicals from secretory glands into the air. To dispel this possibility, it was confirmed that cilia scale and socket cell nuclei had localized Cut in both male and female butterflies, although females had fewer cilia cells overall (Sup.II.2).

Many other bristle-like scale types exist throughout butterflies, with the most prominent examples being in the glasswing butterflies and *Graphium* swallowtails where large patches of wing are transformed from the typical flattened scales to much more slender forms that reveal the either transparent or vibrantly colored wing membranes, respectively. (Sup.II.3). Cut staining was tried in these species to confirm whether the association between its nuclear localization and cylindrical scale shapes remained in other nymphalid tribes, for the ithomiine glasswings, and in other butterfly families, for the papilionid *Graphium* swallowtails. Cut localized to the nuclei of bristle-like cylindrical scales in *Greta oto*, but not to those of either forked or flattened scale types and was expressed early in scale growth (Fig.II.3). This held true for *M. confusa* glasswings, which had Cut localization only in the transparent window region cylindrical cells and not the Pacman-shaped or flattened scales (Fig.II.4). At early stages of development, these non-cylindrical scale types appear identical but can be differentiated by the former always emerging in close proximity to the cylindrical scales. *Graphium agamemnon* on their dorsal wing patches are covered in peg-like scales and, directly opposite on the other surface, the ventral wing patches sport elongated scales that more resemble *Junonia* cilia. These ventral cilia-like scales were found to localize Cut in contrast to the dorsal peg-like scales and all flattened scales which were Cut-negative (Fig.II.5). For all species, Cut was localized along the wing edge defining the imaginal wing shape and in the sensory scale nuclei (data not shown).

Scanning electron microscopy of the Cut-positive scale types confirms that they are cylindrical in shape and reveals that they all have circumferential ridges (Fig.II.6). This condition is referred to as “abwing-ized” as it represents the universal presence of abwing scale features with an absence of any indication of the adwing smooth laminal surface of a flattened scale. This characteristic is directly reminiscent of *Drosophila* and other insect bristles and setae which are similarly cylindrical with circumferential ridges, demonstrating that these cylindrical scales are on the extreme end of the bristle-scale morphotype continuum. Interestingly, although initially appearing to be cylindrical, since the *Graphium agamemnon* dorsal peg-like scales have a flattened lower lamina they are more akin to miniaturized scales severely limited in their widening and extension. This suite of characters is shared by that of “scale-hairs”, semi-cylindrical setae that retain a flat adwing laminal surface (Fig.II.6D’) (Kellogg 1895).

II.3.3 Cylindrical scales have less reticulated microtubule networks

From the developmental series of cytoskeletal organization in fixed *P. xylostella* scales, it is apparent that the microtubule network undergoes significant restructuring throughout scale growth (Fig.II.1). Live imaging of *J. coenia* scales captures this dynamism, visualizing the initial dense occupation of the budding scale in initial projection, parallelized orientation as first extension occurs, and subsequent rapid uncoupling to produce a microtubule-dense basal region at the pedicel with a parallelized arrangement and a reticulated network distally towards the elongating scale tip (Fig.II.7). By contrast, in the development of *Greta oto* scales in a

transparent “window region”, the flattened scales almost immediately after initial projection adopt the microtubule architecture outlined for *J. coenia* scales—a diffused, reticulated network distally—but the developing cylindrical bristle-like scales at this time point, even though they project from the epithelial surface before the flattened scales, maintain a parallelized arrangement of microtubules (Fig.II.8).

In these early growth stages, the persistent coupling of actin and microtubule networks, or, in other words, the continued parallel arrangement of the microtubule cytoskeleton, is a hallmark of cylindrical scale types. In the cilia of *J. coenia,* bristle-like scales of *Greta oto* and *M. confusa*, and the ventral cilia of *Graphium agamemnon*, microtubule density is higher throughout the length of the scale projections, while neighboring flattened scales have lower microtubule density distally most likely as a result of reticulation (see reduction in intensity as the microtubules get reorganized into a more diffuse, network-like arrangement in Fig.II.7, Fig.II.8, and especially Fig.II.10). Even in the miniaturized peg-like scales the microtubules have lower density distally, along with a lack of overlap with the actin cytoskeleton, unlike as is the case for the ventral cilia which have the cytoskeletal networks overlapping throughout the majority of their length (Fig.II.9D&E’).

II.3.4 Stabilizing microtubules during scale flattening recapitulates cylindrical growth

It is not clear from the correlation between microtubule organization and cellular architecture which way the arrow of causality is pointing. Do more parallelized microtubules shape the cell into more cylindrical growth or do microtubules follow the morphology of the cell and their organization is downstream of a truly causal factor that determines scale shape? Live imaging allows observing the effects of pharmacological manipulations on cell growth pattern to more precisely assign causality to experimental manipulations. Live *P. xylostella* microtubule dynamics mirror those of *J. coenia*, as suggested by (Fig.II.1), with the initial uniform filling of the first projection of the scale bud falling away to successively more diffused and reticulated organization distally as flattening proceeds, with just a remnant high concentration of parallelized microtubules overlaying the actin bundles in the pedicel region (Fig.II.10). After the introduction of the microtubule-stabilizing cancer drug taxol, scales in *P. xylostella* in the flattening phase halted reticulation of the microtubule cytoskeleton (Fig.II.11). These pharmacologically treated scales switched from a flattened to a cylindrical growth form to produce spear-shaped final morphologies and parallelized microtubule cytoskeletons in the regions grown after drug introduction (Sup.II.4; Sup.II.5).

Because different scale types emerge in sequence and complete their development at different times, treatment with drugs can affect neighboring scales at different stages with the fortuitous upside of showing differential effects of drug treatment throughout development in the same individual. Cover scales that had already expanded and flattened did not show an effect on morphology from taxol treatment, whereas middle scales hit before the flattening stage became slender and cylindrical in profile, often with inflated tips, and ground scales affected by taxol as they first emerged were completely bristle-like and extremely slender (Sup.II.6A). The microtubule cytoskeletons of these sequentially affected cells reflected their morphology, with the ground and middle scales having concentrated, parallelized tubulin staining throughout their

length, and cover scales having the diffuse distal reticulated pattern of flattened morphologies. In some cases, heterogeneity in microtubule organization as it relates to scale flattening along its length can be observed, with a diffuse section of microtubules flanked on either side by more parallelized networks and respective change in form from cylindrical, to flared out, and back to cylindrical along these transitions (Sup.II.6B).

II.3.5 ACME fixed dissociation can separate pupal wings into single cells for scRNA-seq

Live cell dissociation can introduce artifacts into expression data if samples are not processed quickly as a result of transcriptional programs relating to stress being activated upon disintegration of the inter-cell junctions or to exposure to hypoxic conditions (Massoni-Badosa *et al*. 2020; Ascención *et al*. 2021). To avoid this, flash freezing of cells followed by fractionation to recover just their nuclei for sequencing has become a popular alternative (Fischer & Ayers 2021). However, this approach loses any transcripts which may exist in the cell body, which could potentially be problematic for high volume, polyploid cells like scales that have extensive cytoplasm with very active trafficking of vesicles (Chapter I). Fixing the wings as soon as possible to lock in the transcriptional state while also being able to retrieve single, intact cells reduces the risks of live tissue dissociation by extending the time needed to complete the dissociation and allowing storage of cells over longer terms in the freezer for later multiplexing if cell counts are low. Using ACME fixation-dissociation, wings were completely dissociated into single cells while maintaining high cellular integrity as evidenced by initial budding scale processes remaining attached to the cell (Fig.II.12A,B). Applying this protocol to *G. oto* demonstrates its flexibility and potential for comparing expression at the cellular level between species, at least in the Nymphalidae (Fig.II.12C). Of all cell types that could be distinguished in the single-cell suspension, scale-socket pairs were particularly affixed to each other and were the first to not separate if mechanical forces in the nutation step were not sufficient. Regardless, by committing to vigorous pipetting of the wing tissue, especially as it first enters the ACME solution, the majority of these pIIa daughters separated. An initial sequencing run of dissociated *J. coenia* scales at the initial projection stage produced favorable metrics regarding the number of cells, mean reads per cell, and valid unique molecular identifiers in the sample (App.II.I).

II.4 Discussion

Microtubule architecture is highly dynamic in scale growth and modifications to its structure have been implicated in this study to differentiate between flattened and cylindrical morphotypes. Differential uncoupling of the actin and microtubule cytoskeletons correlates to whether or not scales flatten, both generally and in specific elements of the scale anatomy. For instance, in all scales, microtubules were found to be parallelized along the actin bundles running out of the cell body into the pedicel, which is cylindrical to fit within the socket cell. Sensory scales, which are extremely short cylindrical processes, likewise have microtubules running parallel along the distal-basal axis alongside actin. Wing edge scales, too, have overlapping actin and microtubules along portions of the shaft that are more cylindrical and specialized scale types which resemble bristles, in that they are cylindrical and circumferentially ridged, extend parallel microtubule networks along their full lengths (Fig.II.1B; Fig.II.9). All scales undergo a period in their

ontogeny during initial projection and first extension where microtubules are parallel before becoming a reticulated network during flattening, and by altering the time when decoupling occurs, different scale outlines can be produced—as experimentally drawn out when pharmacologically stabilizing the microtubules during the flattening stage so they could not rearrange resulted in scales recapitulating a cylindrical growth regime and adopting distally contracted, spear-like phenotypes (Sup.II.4; Sup.II.5).

Many cellular systems show examples of such actin and microtubule crosstalk (Dogterom & Koenderink 2018; Pimm & Henty-Ridilla 2021). Coupling of microtubules and actin is known to occur in elongating neuronal growth cones, where such an arrangement facilitates accelerated growth of actin filaments through the recruitment of formin to microtubule plus ends by the linker CLIP-170 (Forscher & Smith 1988; Henty-Ridilla *et al*. 2016). The overlap in these two cytoskeletal polymers in early scale growth may then be a way to accelerate elongation. Scales on the wing must elongate in a specific time window, and thus accelerating the rate of growth of certain scale types is a simple way to introduce morphological heterogeneity along with staggering initial projection times of neighboring cells. Supporting this point, cilia scales in *J. coenia* both start elongating earlier and elongate faster than their flattened neighbors (Fig.I.9).

Similarly, microtubule presence at the cell edge facilitated by its crosslinking with actin can expedite elongation through the rapid delivery of cargo required for cell membrane expansion, like membrane vesicles (Schmoranzer *et al*. 2003; Müller 2019). Coordinated architecture may also adjust forces in the cell projection to the same effect, through the stiffer microtubules stabilizing the leading edge and physically pushing it forward, as is the case in migrating cells (Bouchet & Akhmanova 2017; Etienne-Manneville 2013). In fact, scale buds during the initial projection phase of growth highly resemble a migratory cell on a stiff substrate, with a wide and uneven leading edge as filopodia extend in different directions before polarizing into a unified front (Fig.II.1).

Heterogeneity in microtubule stability, therefore, has significant consequences to cellular form. Although so far attention has been centered on the microtubule arrangement of specialized cylindrical scale types, not to be forgotten is the subsequent decoupling of microtubules from the actin in the case of canonical, flattened phenotypes. This ontogenetic rearrangement presents a scenario whereby the degree of stabilization of the microtubule network has direct readouts to position along a piliform-lamelliform scale axis.

Its correlated localization to piliform scale nuclei suggests a role for *cut* in maintaining a program of microtubule stabilization (Fig.II.2-5). The presence of Cut is known to act as a transcriptional repressor either by active mechanisms or competitive interactions with cell-cycle specific binding activity (Mailly *et al*. 1996; Coqueret 1998; Nepveu 2001). Therefore, in the case of growing scales, the presence of Cut would prevent the transcription of genes whose products are involved in the process of decoupling the actin and microtubule cytoskeletons, maintaining a parallel microtubule array. scRNA-sequencing of these cells appears to be feasible and would reveal differential activation in these Cut-positive cylindrical scale populations relative to flattened scale types (Fig.II.12) (Prakash *et al*. 2023 preprint). In lieu of this

transcriptional data, there are particular candidates involved in regulating actin-microtubule interactions that may play a role in the developmental shift from cylindrical to flattened forms.

Even a single crosslinker can mediate cytoskeletal interactions like coordinated growth and alignment of actin and microtubules (López *et al*. 2014). Actin-binding proteins such as the *Drosophila* spectraplakin-family member *shortstop* work at the cell cortex to mechanically crosslink microtubules to the actin cytoskeleton for functions related to cell-cell adhesion and connecting to the extracellular matrix (Applewhite *et al*. 2013). This activity has consequences for proper nervous, muscular, and respiratory system development (Bottenberg *et al*. 2009). Septins have been termed the fourth structural polymer, and work in the opposite direction of spectraplakins to tether the actin to the microtubule network (Mostowy & Cossart 2012). These mediators have been shown to capture actin filaments on microtubule lattices from which they can extend and promote secondary structures, like the fanning tip of elongating neuronal growth cones (Konstantinos *et al*. 2022).

Cytoskeletal polymers can also have their growth inhibited or be severed. Microtubule-severing proteins are ATPases that bind to microtubules and ultimately encourage their breakdown through several mechanisms (McNally & Roll-Mecak 2018). Kataninlike-1 binds microtubule tips to destabilize them and promote catastrophe (Sonbuchner 2010). Other types, like Spastin, Katanin, and Fidgetin, bind at intersection points of two microtubules and sever one of the pair along its length (Roll-Mecak & Vale 2005; McNally & Vale 1993; Mukherjee *et al*. 2012). If cellular conditions are appropriate, these severed microtubules can then be bound to novel partners in a different orientation, facilitating the formation of new cross-linkages (Wightman & Turner 2007). Functionally, microtubule-severing proteins are important in generating cortical microtubule arrays in plants and rearranging the cytoskeleton throughout animal cell migration (Chi & Ambrose 2016; Zhang *et al*. 2011). There are likewise actin-severing proteins, which follow roughly the same logic, that are composed of such factors as cofilin, which is ubiquitous in cellular rearrangements related to motility and cytokinesis, and gelsolin, which severs actin filaments in the presence of calcium signaling (Pollard 2016; McGrath *et al*. 2000). Such cytoskeletal rearrangements in relation to external signaling or cell state are common, and, in cases when not detected by the severing factors themselves, are conveyed through integrating factors (Sacks 2006). Some of the most notable and far-reaching integrators in this respect are the IQGAP proteins which mediate cell receptor-mediated signaling, external factors like protein kinases and β-arrestins, and the cytoskeleton, effectively tying actin and microtubule networks with the cell signaling environment by acting as an adaptor between their constituent polymers and other pathway factors (Thines *et al*. 2023; Cao *et al*. 2015). IQGAP1 has also even been demonstrated to crosslink actin itself directly (Fukata *et al*. 1997). These integrating factors have important implications for health and morphogenesis, as it opens up the possibility for cytoskeletal crosstalk with any number of physiological states (Hedman *et al*. 2015).

Since both microtubule and actin cytoskeletons are dynamic and go through distinct phases of organization throughout different stages of scale growth, these transitions can be hypothesized as being mediated by stepwise handoffs between crosslinking and cytoskeleton-interacting factors. In the case of a normal flattened scale, when the microtubule cytoskeleton is aligned with actin

growth in the initial projection and first extension stages, spectraplakin-like actin-microtubule crosslinkers can link the two cytoskeletal networks together to increase the speed of elongation. During scale flattening, the actin and microtubule cytoskeletons could uncouple through the activity of a katanin-like mediator of dynamic instability that severs the microtubules such that they reconnect in a reticulated pattern. Once the scale has reached its maximum growth, the cortical actin rearranges to prefigure fine microstructural elements of the adult scale, a process which could be facilitated by the unbundling of actin from rods and subsequent linkage to the reticulated microtubule structure through action of an actor like a Septin. Shifts between these stages could be transcriptionally tied to the cell cycle, or again the result of regulation by cytoskeletal linkers which report cellular state, like IQGAP1. To produce diverse scale types, the activity of a repressor gene like *cut* would merely have to prevent the transcription, translation, or proper localization of the specific stage-defining mediator(s) and in doing so would promote persistence of the previous growth regime.

Despite the elegance of such a proposed model, shifts in cytoskeletal organization need not be, or at least wholly be, mediated by bound factors. Simple differences in the initial cytoskeletal polymer conformational state can have cascading effects due to eliciting differential interactions with binding proteins (Harris *et al*. 2020). Other modifications like microtubule acetylation can also change binding affinities, rescuing them from catastrophe by preventing the binding of degrading factors or producing a distinct population of polymers under its own signaling and control (Nekooki-Machida & Hagiwara 2020; Dompierre *et al*. 2007; Eshun-Wilson *et al*. 2019). This investigation only focused on interactions between two specific classes of cytoskeletal polymers, filamentous actin and acetylated α-tubulin. Distinguishing the filamentous actin isoforms to separately visualize distributions of α- and β-actin could reveal that each prefigures distinct subpopulations of the cytoskeletal pattern described in this work and investigating other isoforms of tubulin like β-tubulin may demonstrate that it prefigures separate processes than described or reinforces these dynamics by sharing their organizational motifs (Dugina *et al*. 2022; Sulimenko *et al*. 2022; Bitan *et al*. 2012). Other forms of connections than crosslinking may also occur, such as actin filaments presenting inside microtubules (Paul *et al*. 2020). This configuration was recently discovered through cryo-electron tomography and there remains no explanatory mechanism for how it occurs, opening the possibility for it to be a purely physical phenomenon outside the influence of binding factors.

Physical forces, too, like polymer curvature and osmotic pressure can influence cytoskeletal kinetics to affect differential binding affinity and slow or speed polymerization rates (Pearce *et al*. 2018; Molines *et al*. 2022). These physical phenomena can often be thought to engage in feedback loops where an initial restructuring due to presence of a binding factor impacts the physical conditions of the cell, for example increasing the cytoplasmic viscosity, and then all cytoskeletal factors start behaving within the new parameters (Chaubet *et al*. 2020). For factors which may be relevant to the dynamic shifts observed in scale cytoskeletons, compression of cells has been found to stabilize microtubule networks (Li *et al*. 2023). If such forces were created in a gradient over the wing by, for example, differing tautness conferred by the lacunal tissue as it undergoes apoptosis and the wing contracts, then these differential forces may

partially contribute to the observed continuous variation of scale type morphologies or differing growth rates between wing regions.

Regardless of the factors acting downstream of *cut* to produce piliform scales, the fact that a single transcription factor correlates with a variety of cylindrical scale types raises questions about the genetic control and evolution of scale form. It is unclear whether there is a piliform scale/bristle program specified by *cut* and certain lineages of Lepidoptera have employed this ancestral pathway to produce a bristle in the wing, as is the case for the transcription factors which define classes of scale color in *Heliconius melopomene*, or if *cut* is used as a cell shape modifier during development, wherein it is expressed in many different scale classes to repress later morphogenetic stages and produce cylindrical scale forms with circumferential ridges (Fig.II.13). The crux of this question is whether Cut-positive cells are undergoing a fate change to effectively become bristles—which would constitute their own evolutionary class of scale type in Lepidoptera—or if cylindrical scale forms are convergent and can result from co-opted Cut localization in the nuclei of any scale cell. Answering this question is difficult but may be resolved if it were possible to ectopically express *cut* in all or a patch of scale nuclei. If all of the scales adopt a bristle-like morphology but retain characteristics of their original type, like color, then *cut* could be thought of as a shape modifier. If, instead, these cells were to all become uniform cilia, then *cut* is likely specifying an ancestral bristle program in the wing, overwriting the previous fate. If the latter is true, then scale shape evolution can be thought of in terms of the selection of specific defined fates, but if *cut* has the hallmarks of a shape modifier, then cell shape exists on a continuous axis that is more flexible and amenable to gradation.

Whether through specifying an ancestral bristle class or altering scale growth patterns, *cut* facilitated the evolution of transparency in glasswing butterflies through shifting flattened scale types to be cylindrical. Similarly, *cut* expression specifying interior wing cilia could allow for the evolution of more efficient pheromone dispersal. These evolutionary shifts can be understood at the level of differences in cytoskeletal regulation because scales are formed from single cells, and so changes to their cytoskeletal architecture can domino to have a direct impact on organismal fitness. In both cases, these alterations to scale cytoskeletal organization can be linked to direct fitness impacts to the organism and influence interactions with other individuals, species, and the environment. The investigation of genetic factors potentially determining shape differences between structures formed entirely from single cells presents a possibility to understand the mechanisms directly influencing cellular architecture and link them to organismal fitness.

II.5 Conclusions

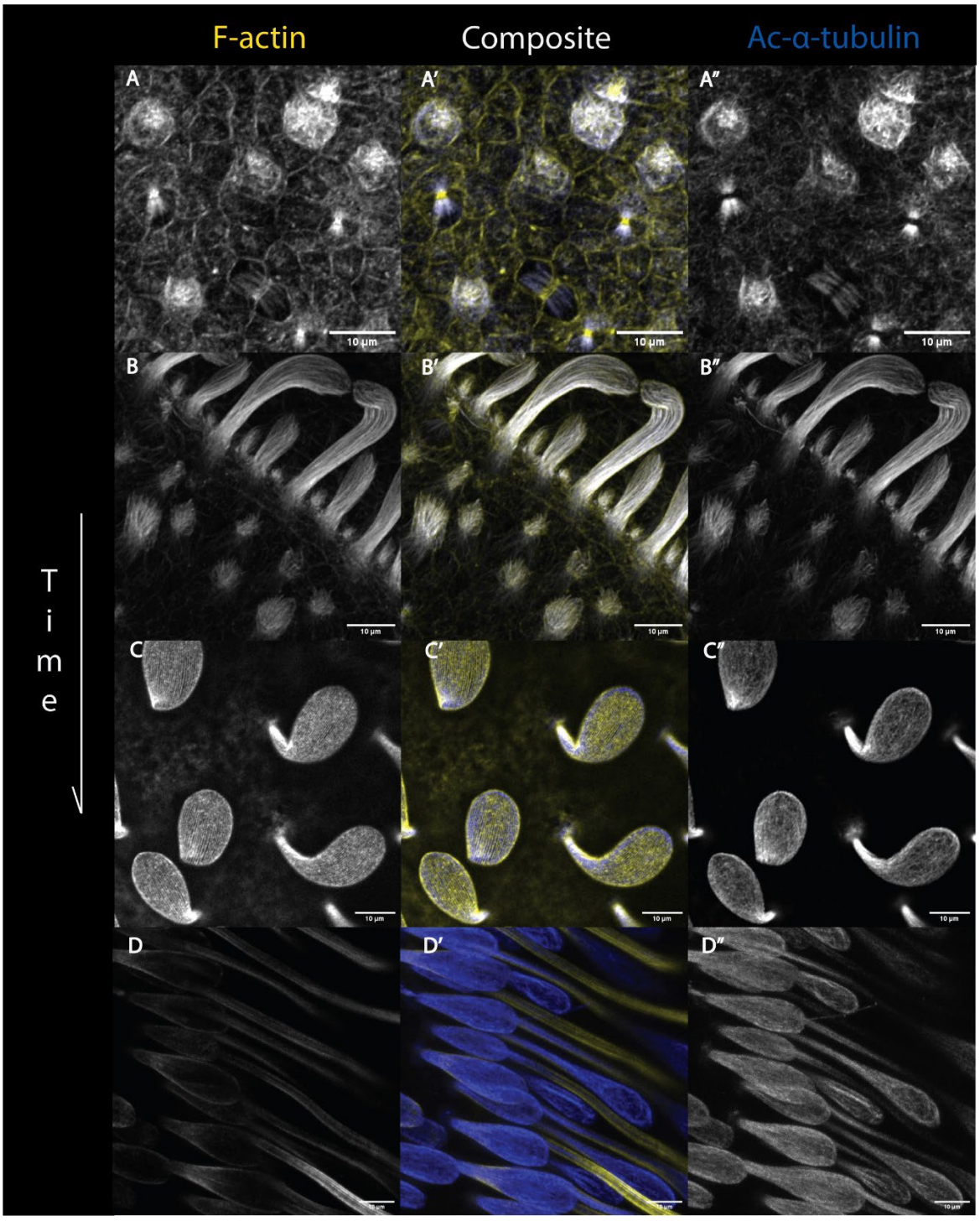
Although a considerable amount is known of the genetic specification of color, factors related to overall scale form have been elusive to pin down as they require coordinated investigation of the cytoskeletal architecture during development along with molecular investigation of transcription factor candidates. Here, I identify a pattern of Cut transcription factor protein localization in the nuclei of cylindrical scale types and make the argument that it transcriptionally represses a

cellular program to uncouple the microtubule and actin cytoskeletons to maintain cylindrical growth and prevent scale flattening.

Live imaging of the cytoskeleton throughout scale growth across species allowed the association between pharmacological manipulations of microtubule stability dynamics and recapitulated cylindrical shape in scales that had already initiated flattening. The power to see changes to morphology in real-time demonstrates the utility of this approach in establishing causal linkages between subcellular organization and cellular morphology, especially when employed in a system like scales where almost the entire anatomy of a structure is determined by the former. Now that the first transcription factor determining a change in overall lepidopteran scale form has been identified, focusing on downstream targets can help to dissect the actual mechanisms underlying differences in growth regime between cylindrical and flattened scale types, and scRNA-seq is a promising next step to understand divergent expression patterns between classes of these morphologically complex single cells. Still, important factors interacting with the cytoskeleton may not necessarily be differentially expressed between scale types. Morphologies may be determined solely by physical phenomena of the growing cell in relation to overall size or proximity to other structures like the cuticle or the extracellular matrix, or just slight variations in accretion or the temporal localization of shared proteins (Tilney *et al*. 2004; Hooikaas *et al*. 2020). Regardless of how factors differentiate or manifest, validation with coincident recording of the cytoskeletal morphology is a requirement to create causal links between candidate factors and phenotypic effects. Therefore, effort should also be invested in developing systems for greater control of cellular architecture through either genetics or the injection of antibodies (di Pietro *et al*. 2021; Zhang *et al*. 2020).

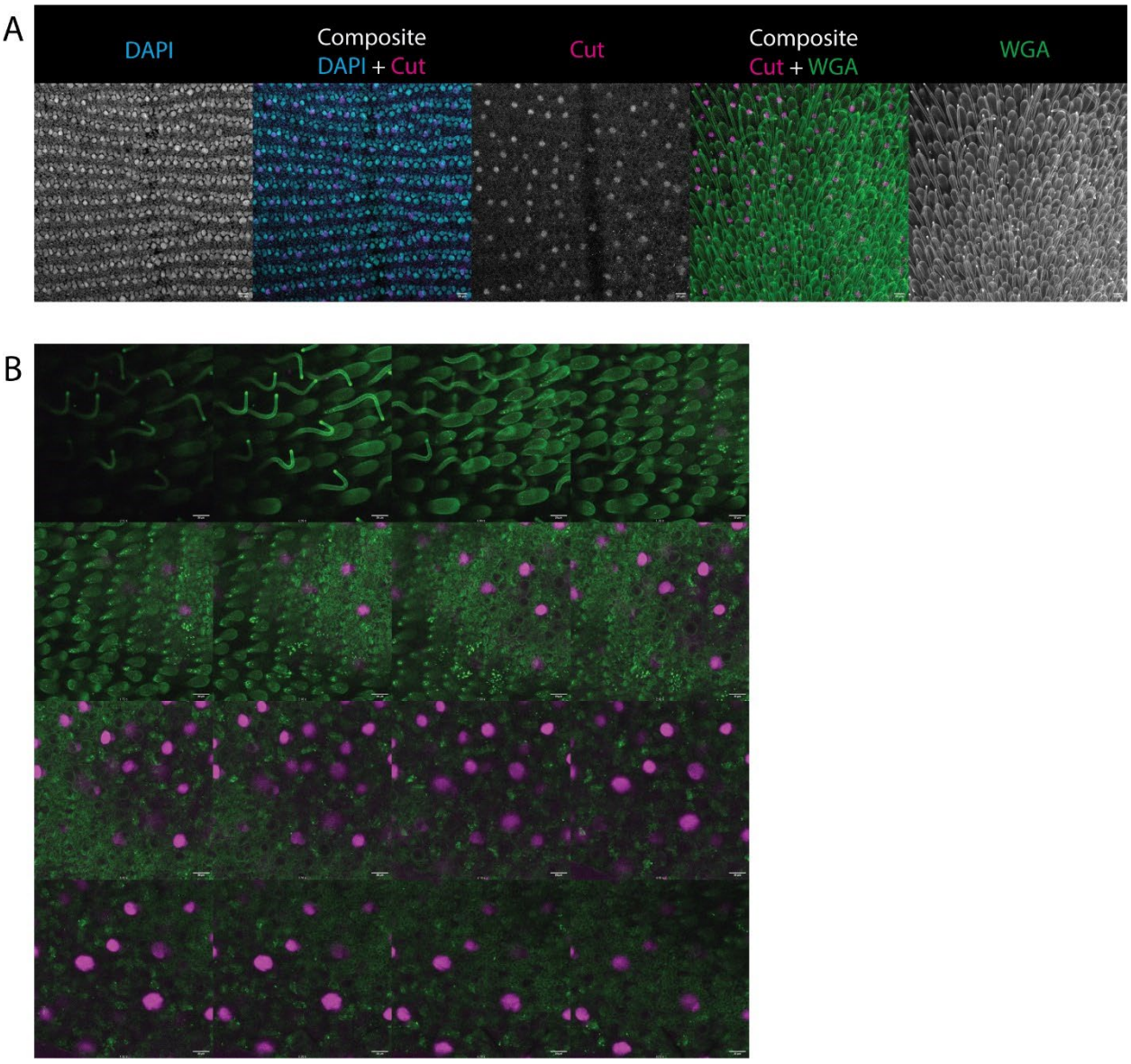
Coordinated regulation of cytoskeletal polymers offers an exciting avenue to understand the diversity of scale shapes that exist throughout the wing. Differences in morphology can likely be ascribed to alterations linking or decoupling the growth of actin and microtubules. It is a tantalizing possibility that temporal regulation of microtubule-actin dynamics is the key factor placing scale morphotypes along the bristle-scale morphotype continuum.

Fig. II.1 Decoupling of the actin-microtubule cytoskeleton in flattened scale growth



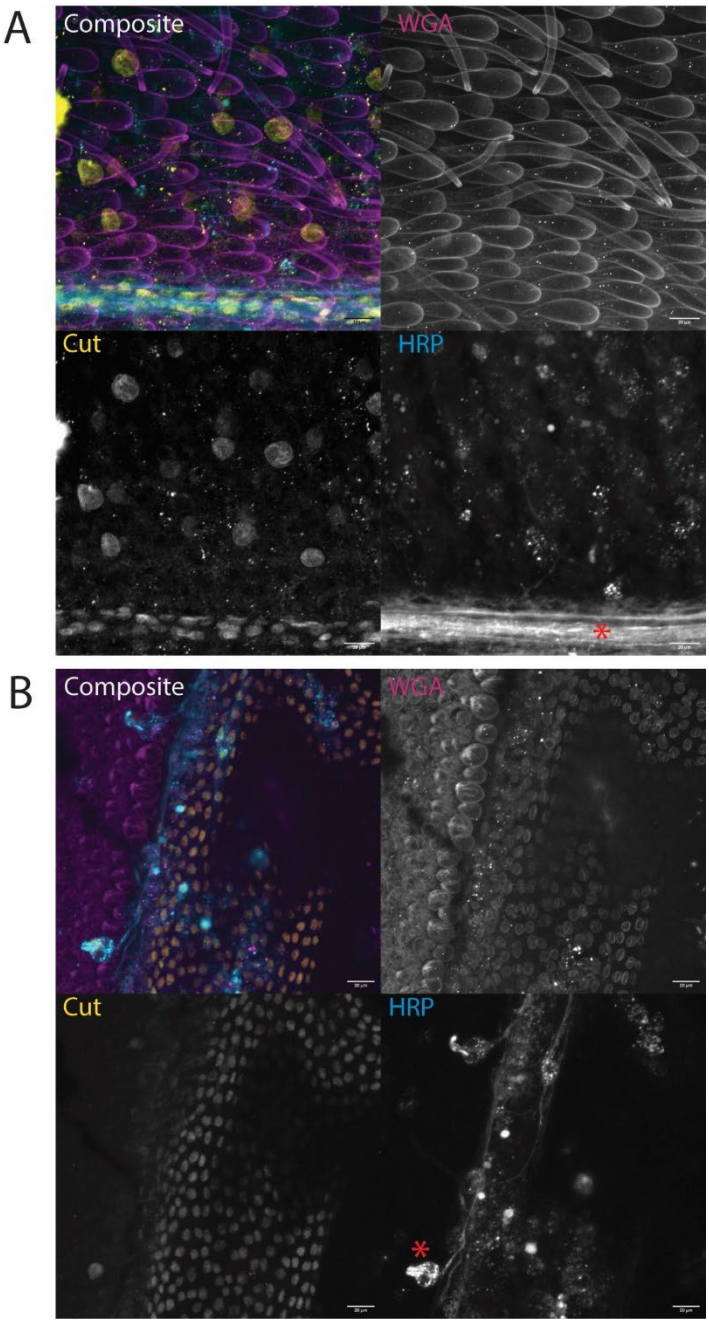
Maximum intensity projections of *Plutella xylostella* ventral forewing scales visualized for the F-actin (X, yellow) and acetylated α-tubulin (X", blue) cytoskeletons and their overlap (X') in initial projection (A), first elongation (B), and flattening (C,D) stages in the wing interior (A,C) and along the wing edge (B,D). Scale bars measure 10µm.

Fig. II.2 Cilia scales in *Junonia coenia* localize Cut protein



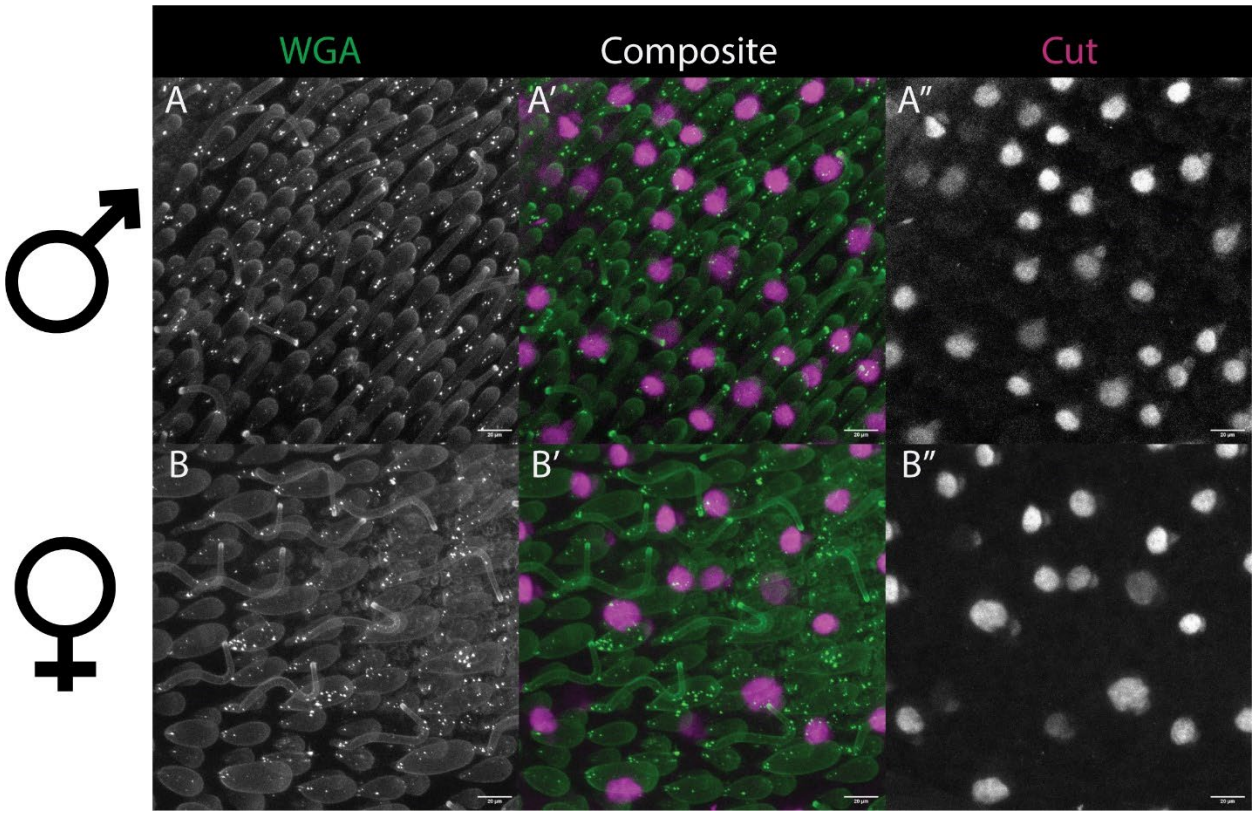
A- Maximum intensity projections of *Junonia coenia* 96HAP dorsal pupal hindwings stained to visualize DNA (DAPI, cyan), Cut protein (α-Cut, magenta), and cell membranes (WGA, green) with combinatorial composites. Scale bars equal to 20μm. Scale distal tips pointing up. **B**- Z-planes through a confocal stack of a 96HAP *Junonia coenia* hindwing stained for cell membranes (WGA, green) and Cut protein (α-Cut, magenta). Scales bars equal to 10μm. Scale distal tips pointing right.

Sup. II.1 *Junonia coenia* cilia scale Cut protein localization is distinct from innervation



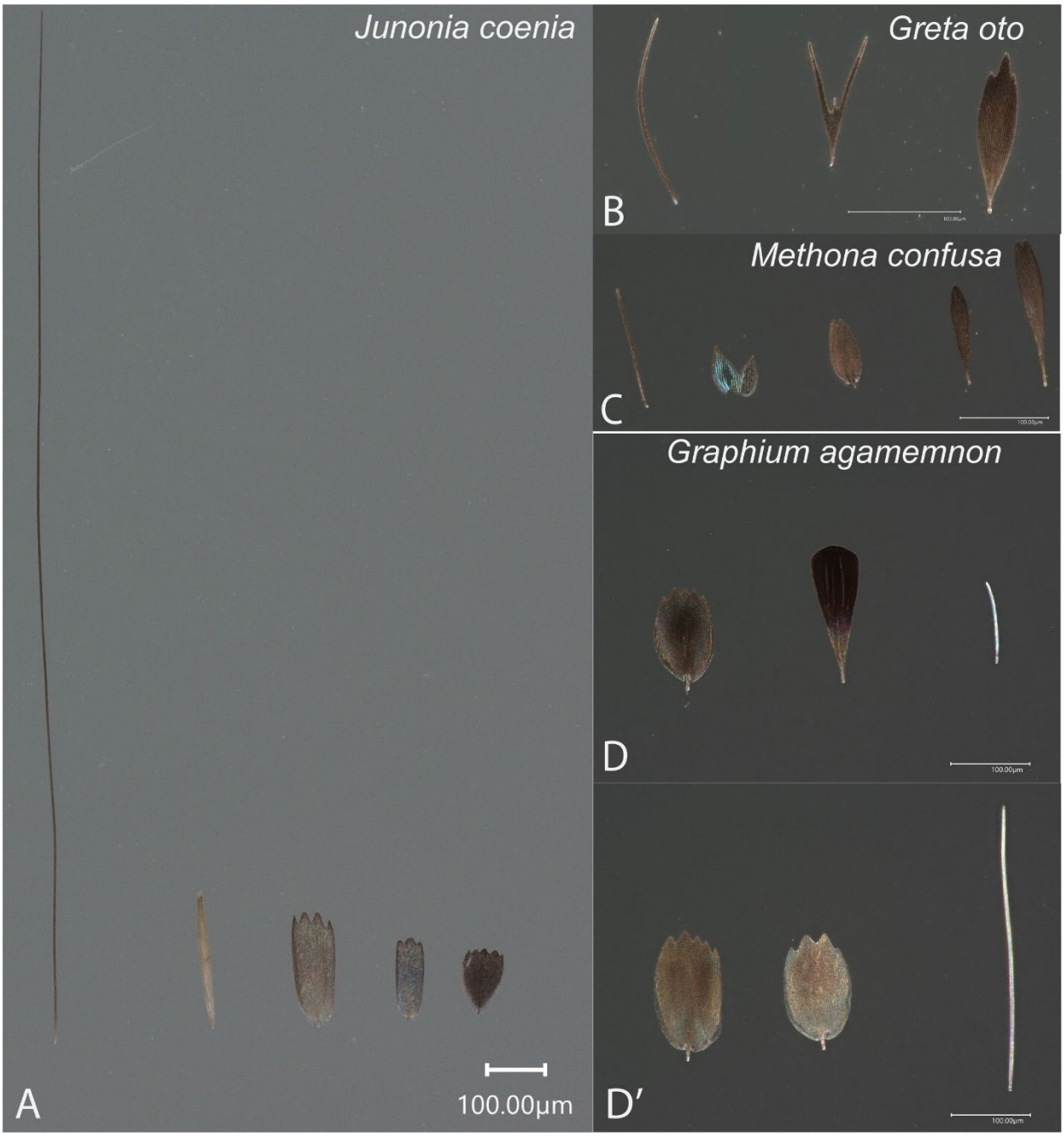
Maximum intensity projections of *Junonia coenia* dorsal hindwings stained for cell membranes (WGA, magenta), Cut protein (α-Cut, yellow), and neurons (HRP, cyan) in the wing interior (A) and along its edge (B). Red asterisks in the HRP panels mark neural projections (A) and sensory organ nuclei (B). Scale bars equal to 20μm. Scale distal tips pointing right.

Sup. II.2 *Junonia coenia* cilia scale Cut protein localization is not sexually dimorphic



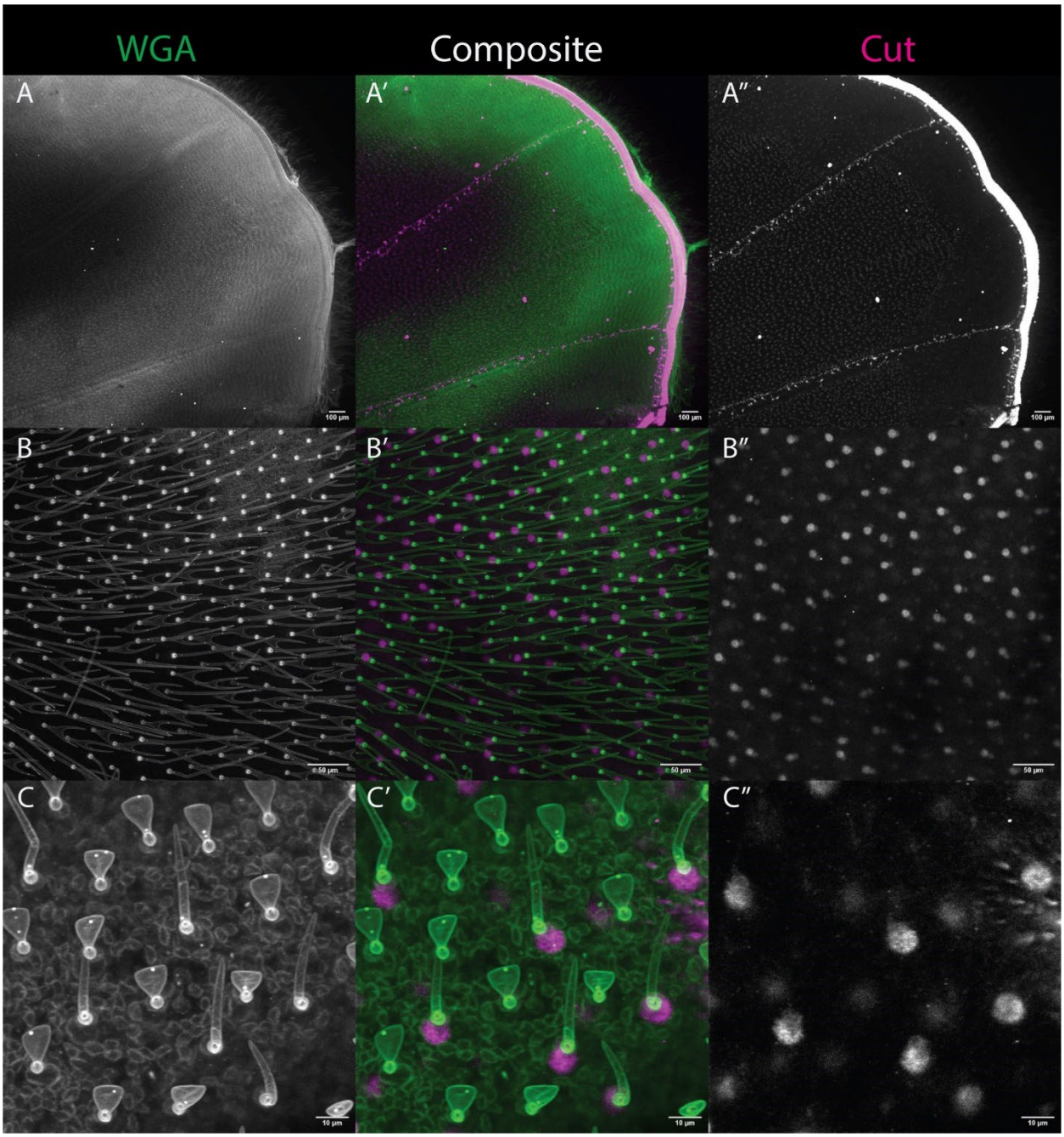
Maximum intensity projections of 96HAP male (A) and female (B) *Junonia coenia* dorsal hindwings stained to visualize cell membranes (WGA-X, green) and Cut localization (α-Cut-X", magenta). Scales bars equal to 20μm. Scale distal tips pointing right.

Sup. II.3 Bristle-like macrochaetae appear throughout Lepidoptera



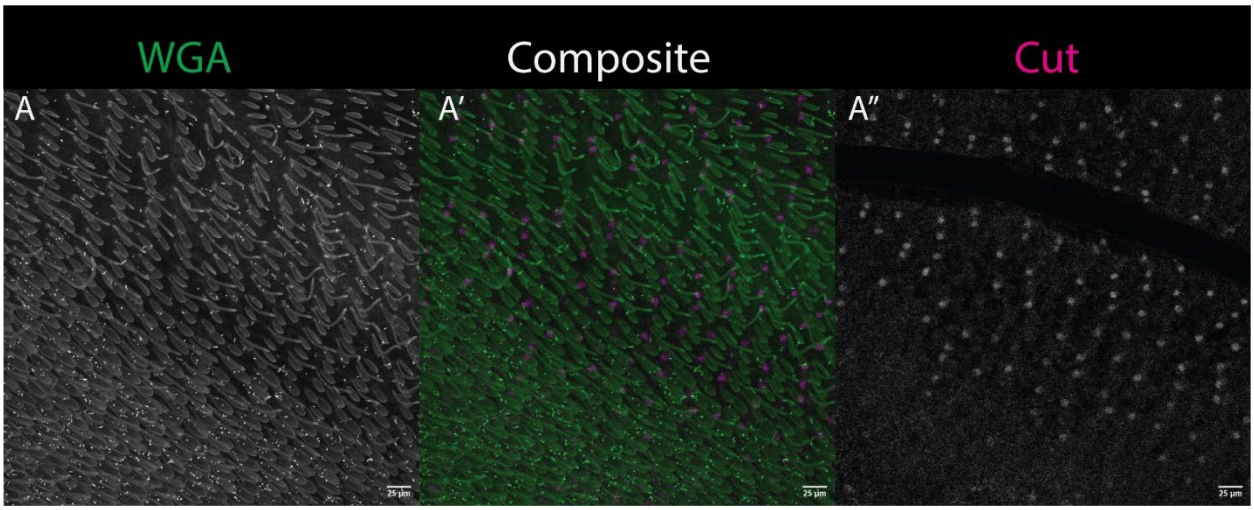
High resolution color photographs of adwing-side-up, individual scales plucked from *Junonia coenia* (A) and *Greta oto* (B) dorsal hindwings and *Methona confusa* dorsal (C) and *Graphium agememnon* dorsal (D) and ventral (D') forewings. Scale bars are 100µm.

Fig. II.3 *Greta oto* bristle-like scales localize Cut protein



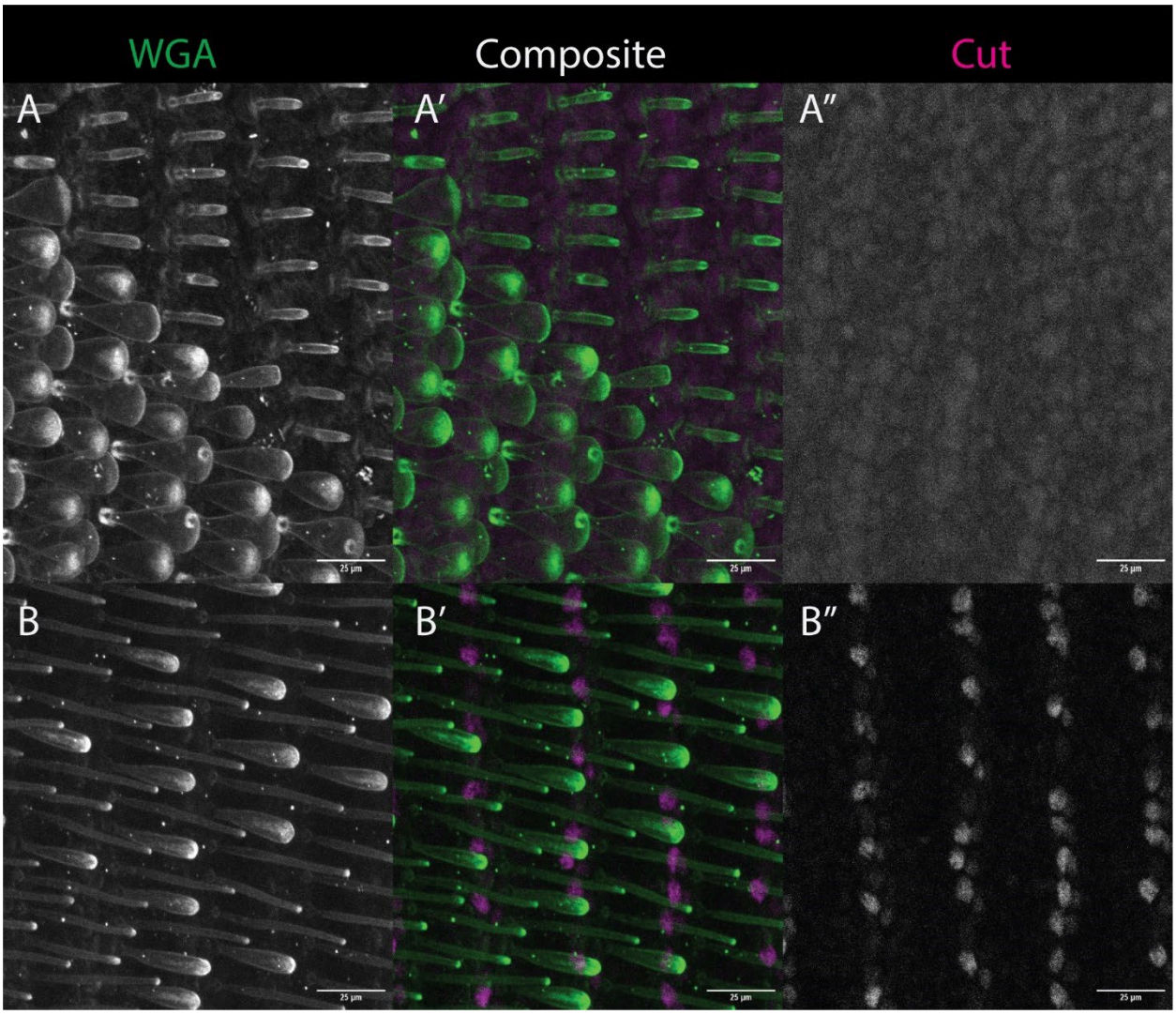
Maximum intensity projections of *Greta oto* 72HAP (A,B) and 48HAP (C) dorsal hindwings stained to visualize cell membranes (WGA-X, green) and Cut localization (α-Cut-X", magenta) in transparent region boundaries. (B) represents almost fully extended scales in a region of mixed forked and bristle morphotypes and (C) represents an earlier developmental timepoint in a region of mixed flattened and bristle scales that are just beginning to extend. Scale bars measure 100μm (A), 50μm (B), and 10μm (C).

Fig. II.4 *Methona confusa* bristle-like scales localize Cut protein



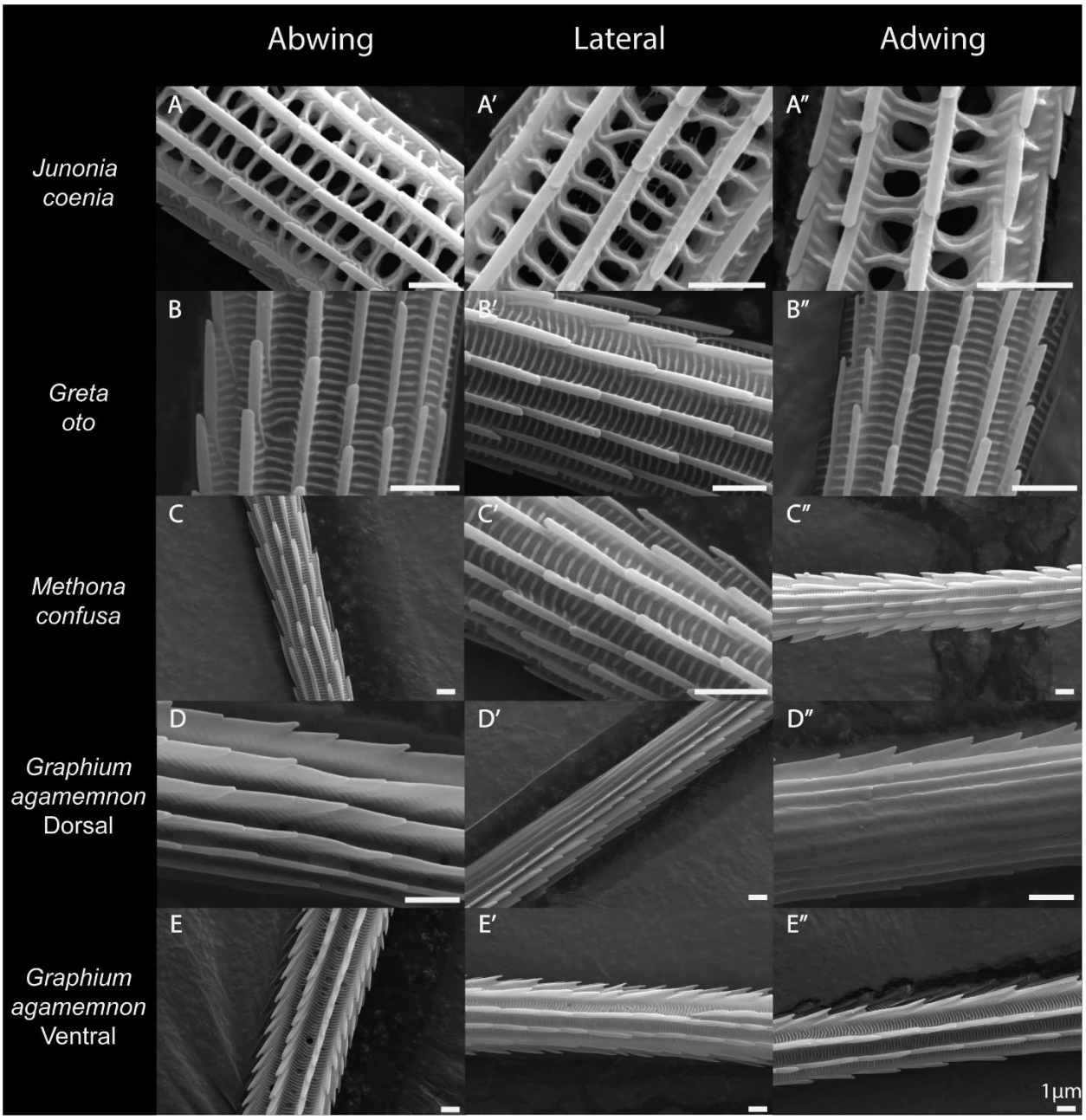
Maximum intensity projections of *Methona confusa* dorsal forewing transparent region boundary stained for cell membrane (WGA-X, green) and Cut localization (α-Cut-X", magenta). Scale bars are equal to 25µm.

Fig. II.5 *Graphium agamemnon* ventral cilia, not dorsal peg, scales localize Cut protein



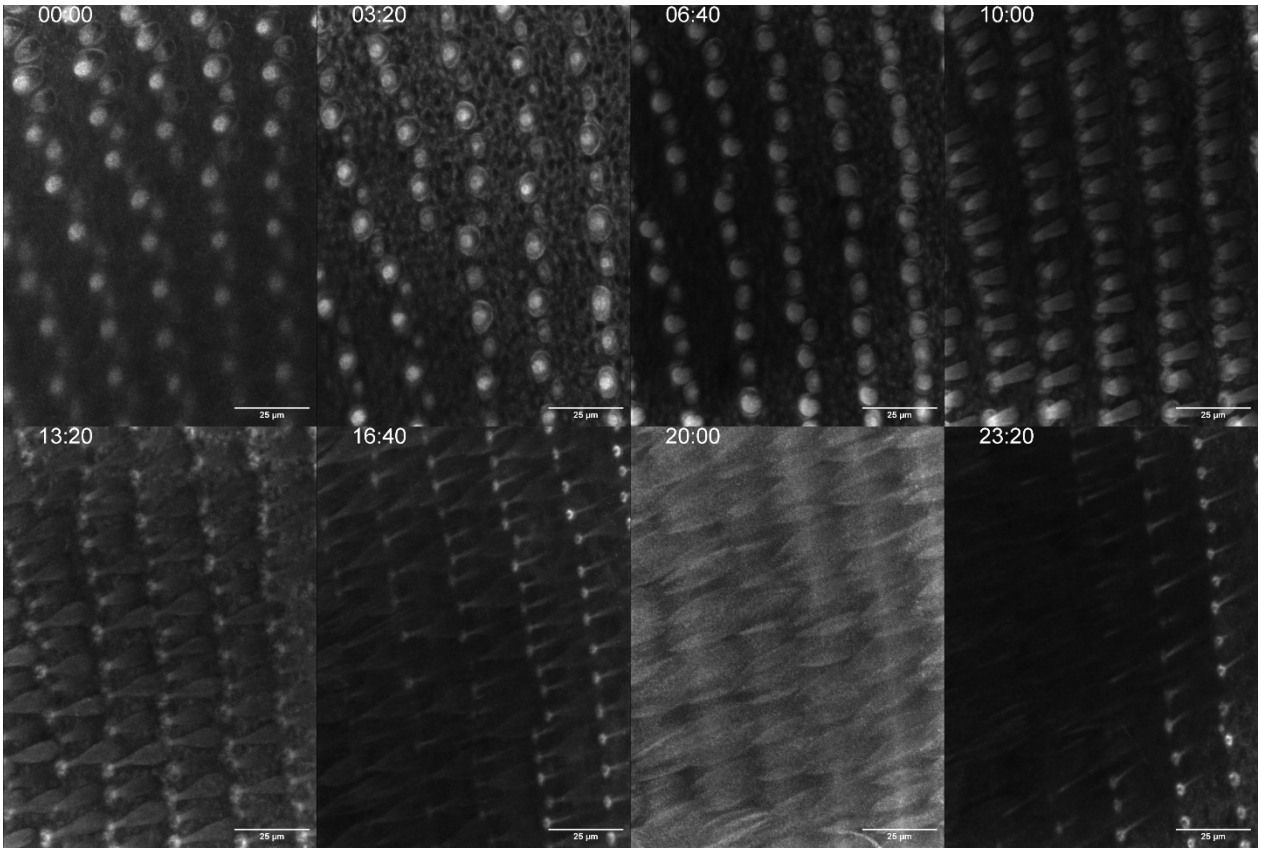
Maximum intensity projections of *Graphium agememnon* dorsal (A) and ventral (B) forewing regions at pigmented membrane boundary regions stained for cell membrane (WGA-X, green) and Cut localization (α-Cut-X", magenta). Scale bars equal to 25µm.

Fig. II.6 Cut-positive scale types share a cylindrical, "abwing-ized" morphology



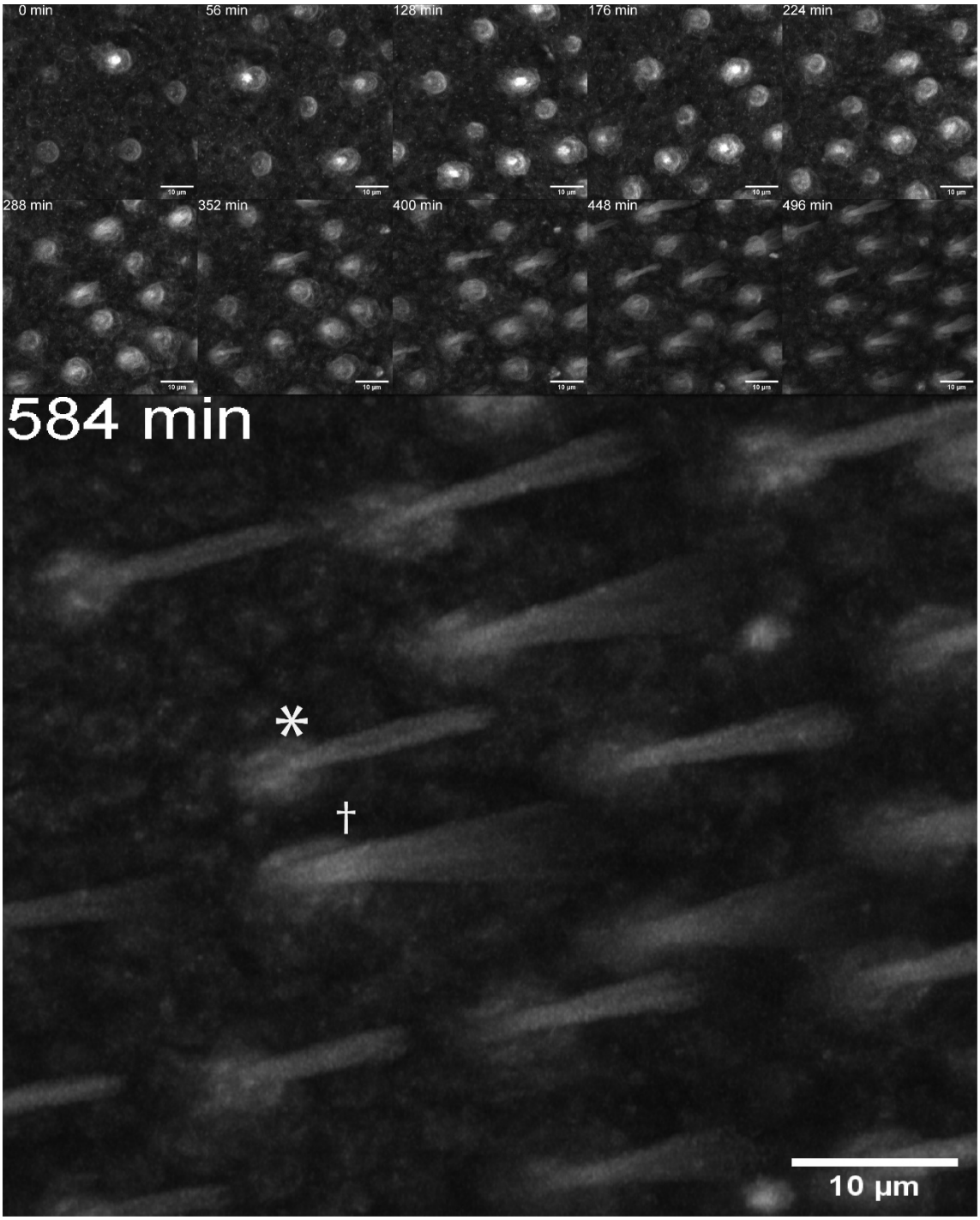
SEM micrographs of *Junonia coenia* dorsal hindwing (A), *Greta oto* dorsal hindwing (B), *Methona confusa* dorsal forewing (C), and *Graphium agamemnon* dorsal (D) and ventral (E) scales' abwing (X), lateral (X'), and adwing (X") surfaces. Scale bars equal to 1µm.

Fig. II.7 Microtubule dynamics in developing *Junonia coenia* scales



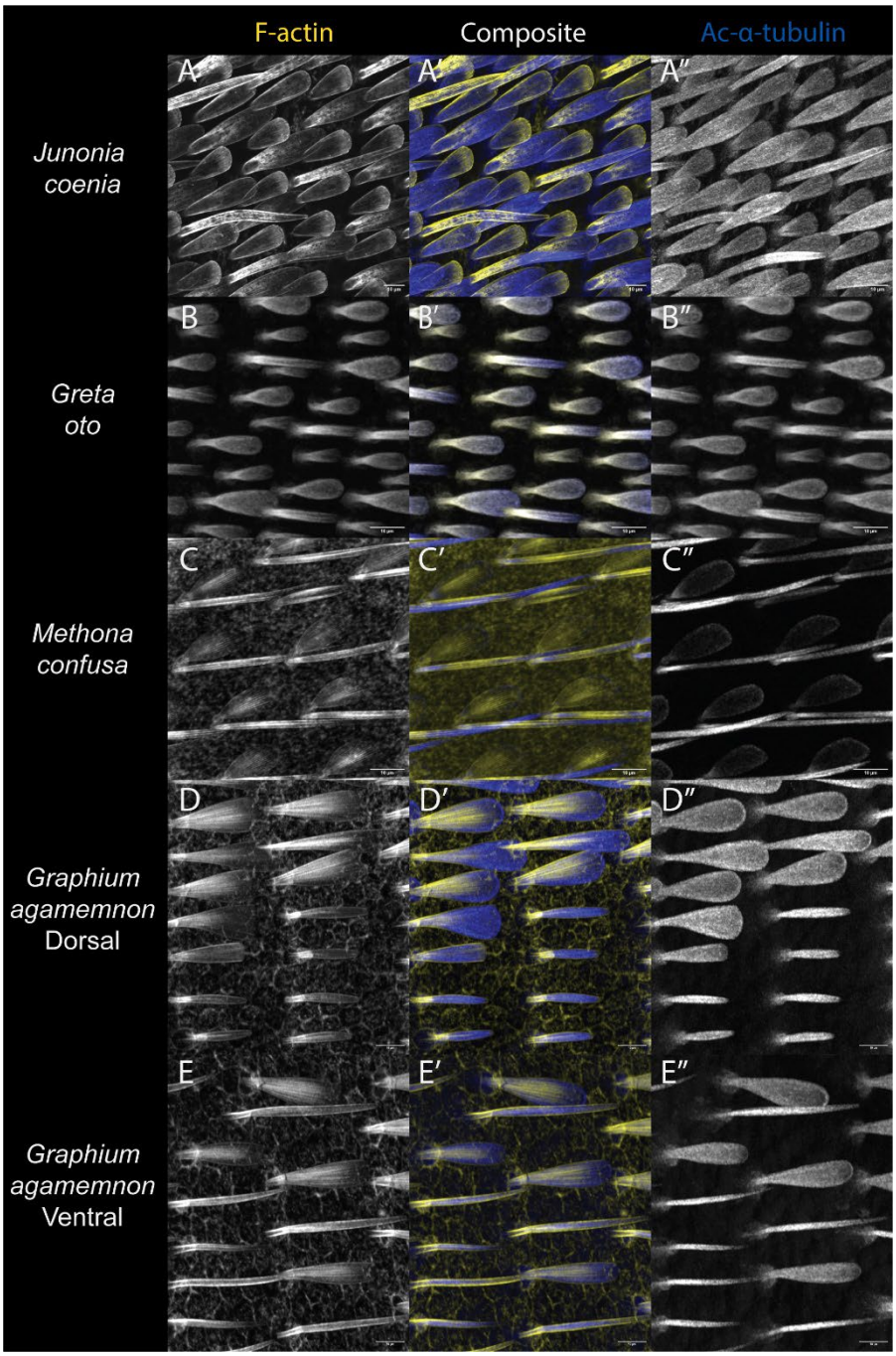
Montage obtained from subsampling a 24hr movie of *Junonia coenia* dorsal hindwing scale microtubule dynamics every 3hr20min where original acquisitions were obtained every 8min, with time represented in hr:min format. Movie time points are maximum intensity projections of acquired Z-stacks with distal scale tips oriented to the right. Scale bars measure 25µm.

Fig. II.8 Microtubules dynamics in early *Greta oto* scale growth



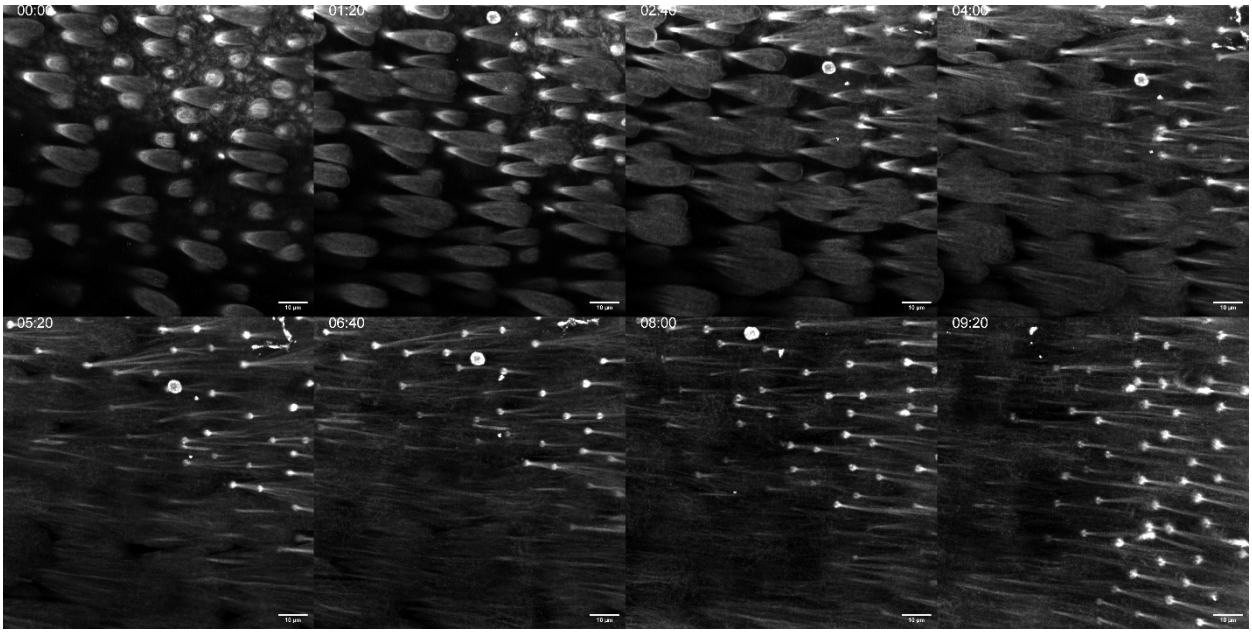
Montage obtained from subsampling an 8hr movie of SiR-Tubulin (microtubules) stained *Greta oto* scales in a transparent window boundary region every 00:56, where original acquisitions were obtained every 8min. The final time point is enlarged to showcase the final microtubule organization of the bristle-like (asterisk) and flattened scales (dagger). Movie time points are maximum intensity projections of acquired Z-stacks with distal scale tips oriented to the right. Time recorded in hr:min. Scale bars measure 10µm.

Fig. II.9 Cylindrical scale types have less reticulated microtubule networks



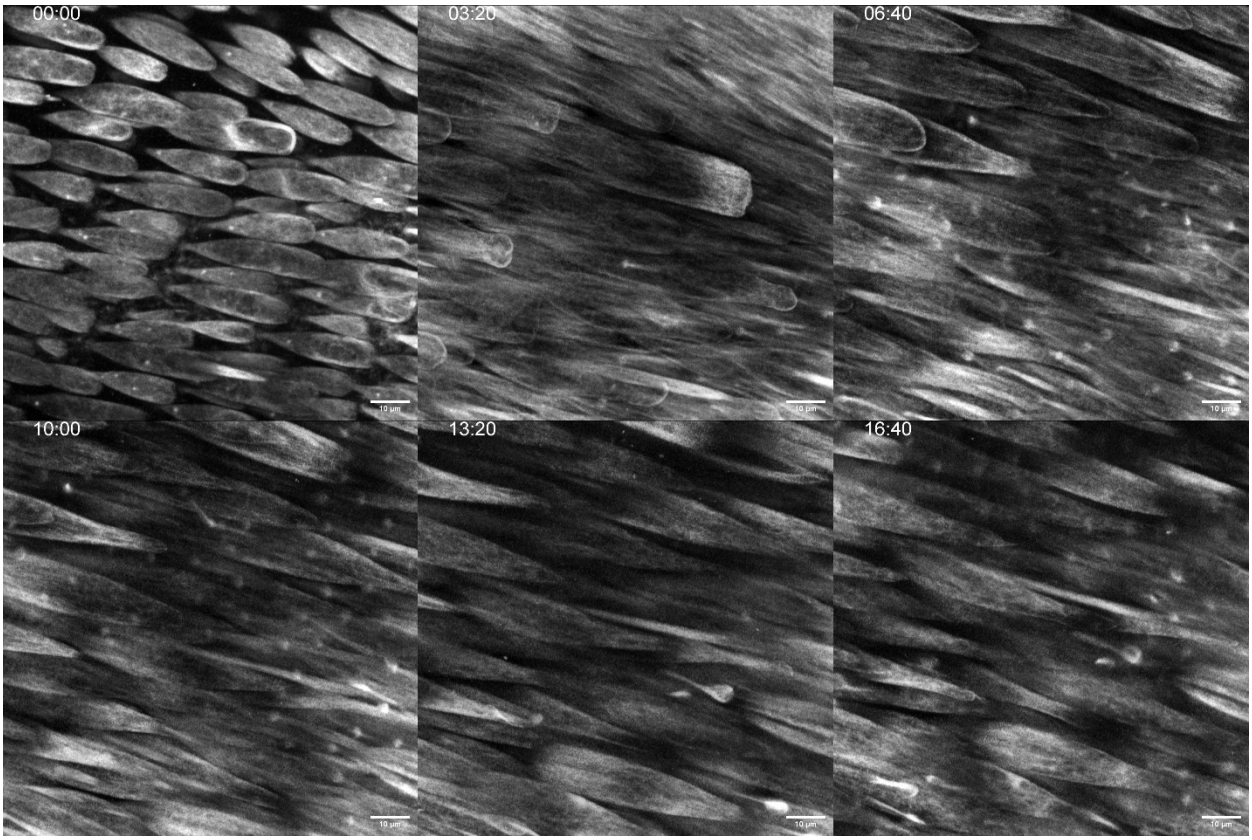
Maximum intensity projections of mixed scale type regions of *Junonia coenia* dorsal hindwing (A), *Greta oto* dorsal hindwing (B), *Methona confusa* dorsal forewing (C), and *Graphium agamemnon* dorsal (D) and ventral (E) surfaces visualized for F-actin (X, yellow), acetylated α-tubulin (X", blue), and their overlap (X'). Cut-positive scale cylindrical scale types are present in (A-C&E). Scale bars measure 10μm. Distal scale tips oriented to the right.

Fig. II.10 Microtubule dynamics in developing *Plutella xylostella* scales



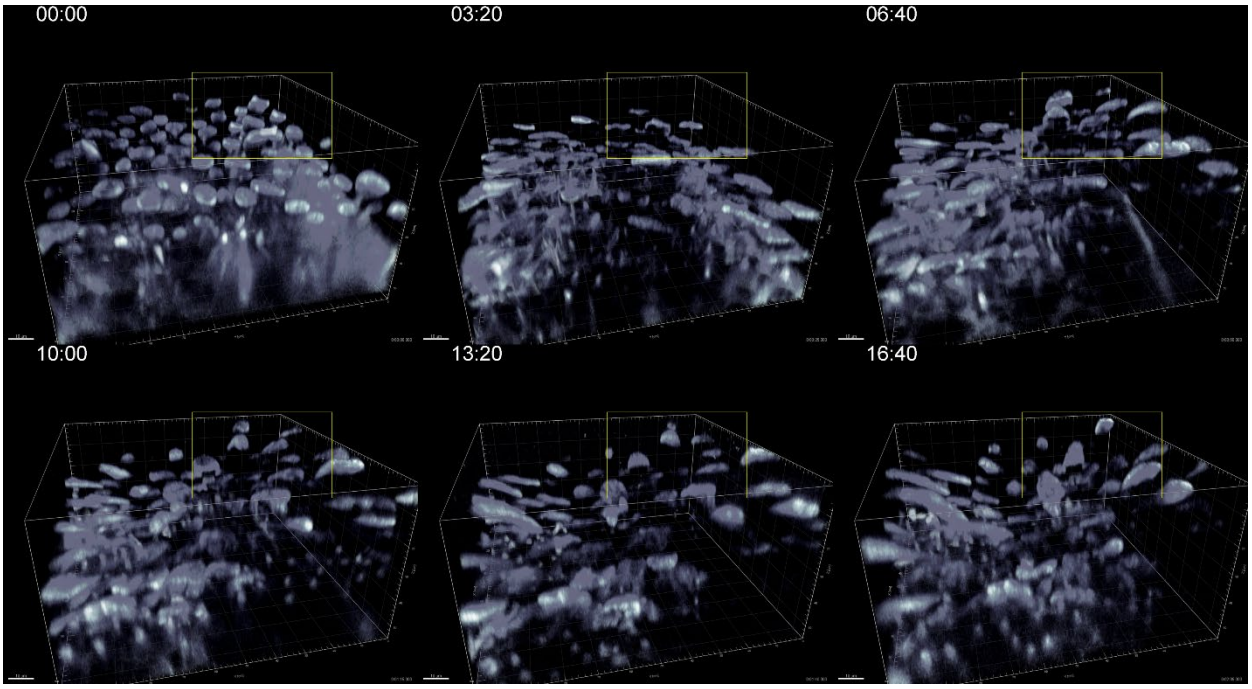
Montage created from a 10hr movie of SiR-Tubulin (microtubules) stained *Plutella xylostella* dorsal forewing scales by subsampling every 1:20, whereas the original acquisitions were obtained every 8min. Movie time points are maximum intensity projections of acquired Z-stacks with distal scale tips oriented to the right. Time recorded in hr:min. Scale bars measure 10µm.

Fig. II.11 Taxol-treated *Plutella xylostella* scales adopt more cylindrical morphologies



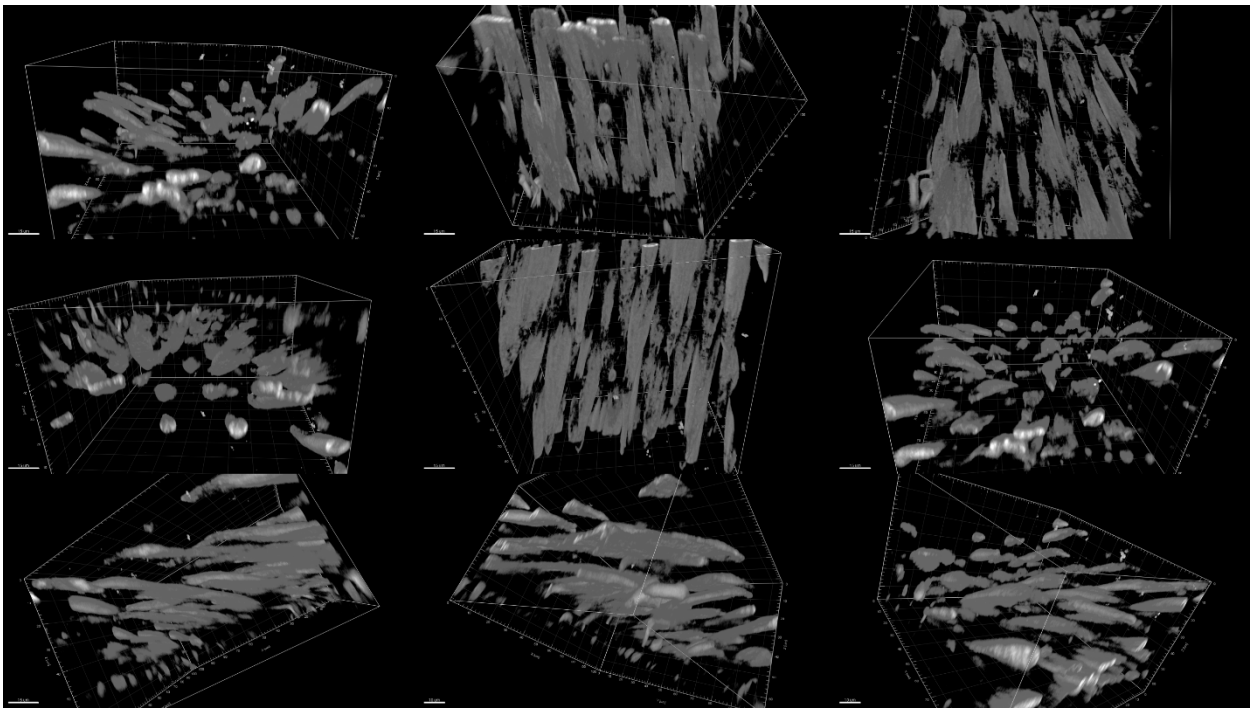
Montage created from a 17hr movie of taxol-teated, SiR-Tubulin (microtubules) stained *Plutella xylostella* dorsal forewing scales by subsampling every 3:20, whereas the original acquisitions were obtained every 8min. Movie time points are standard deviation projections of acquired Z-stacks with distal scale tips oriented to the right. Time recorded in hr:min. Scale bars measure 10μm.

Sup. II.4 Taxol-treated scales switch from a flattened to cylindrical growth mode



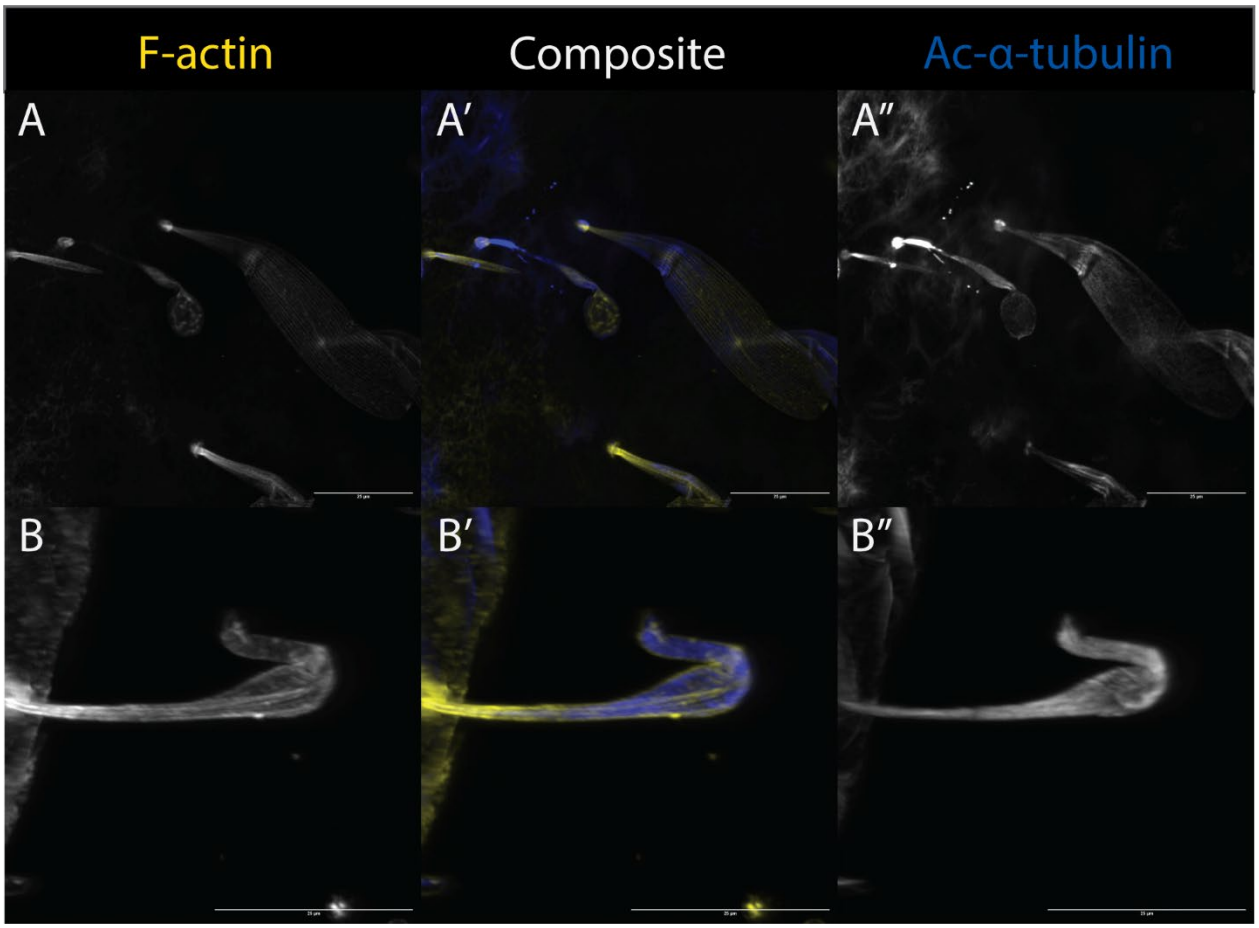
Montage of a 3-D projection of Fig.II.11 with SiR-tubulin (microtubules, grey) stained scales oriented growing towards the viewer to visualize their cross-sections. Panels created by subsampling the full movie every 3:20, where the original obtained acquisitions every 8min. The yellow box indicates a region of particularly obvious transition from a flattened growth habit to a cylindrical one. Time recorded in hour:min. Scale bars equal to 10µm.

Sup. II.5 Taxol-treated scales adopt spear-like phenotypes



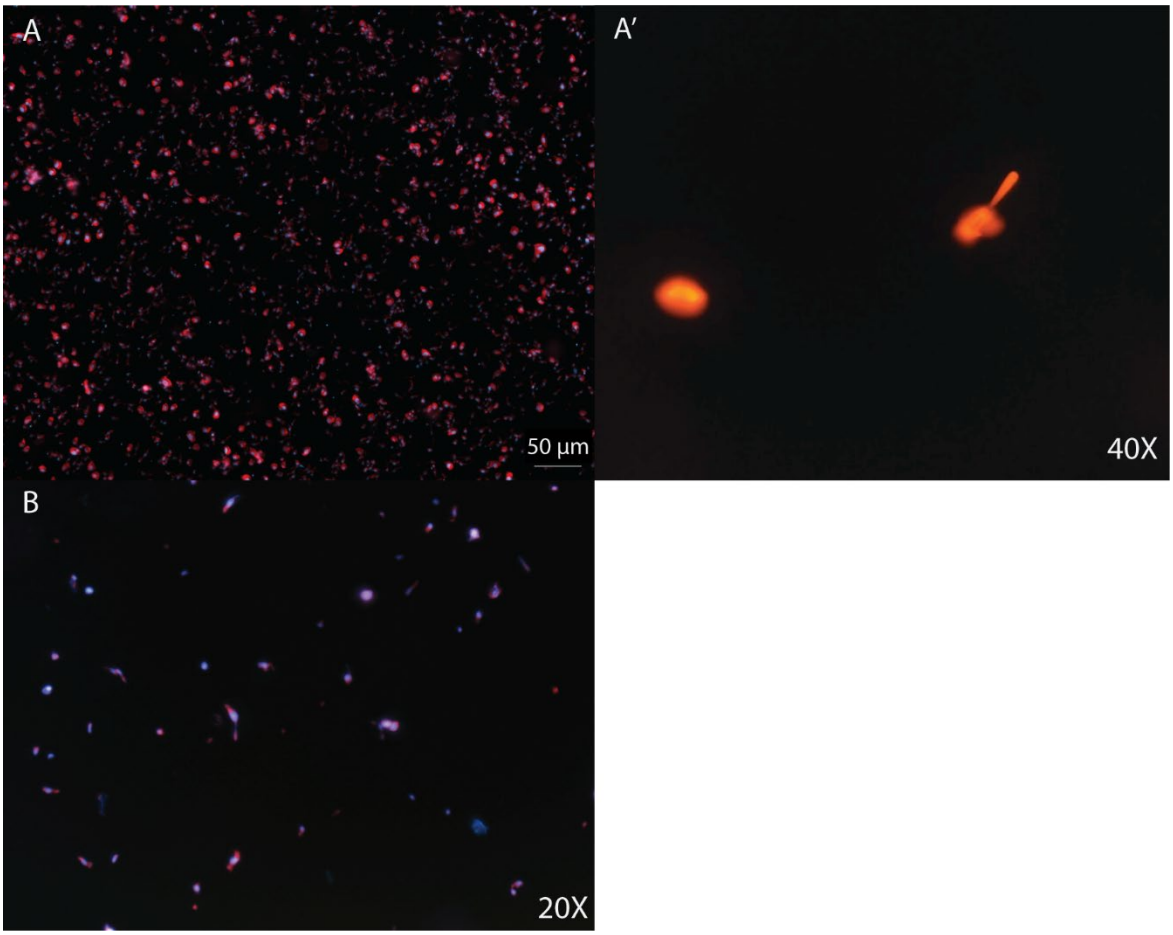
Montage of a rotating 3-D projection movie of the final Z-stack from Fig.II.11 showing different angles of taxol-treated, SiR-Tubulin (microtubules) stained *Plutella xylostella* dorsal forewing scales. Scale bar measures 15µm.

Sup. II.6 Taxol-treated scales have less reticulated microtubule networks



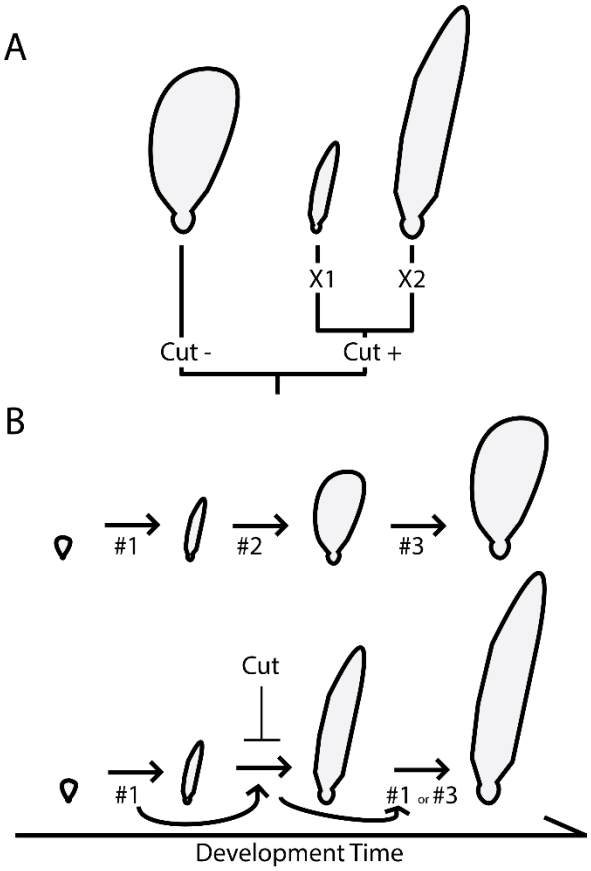
Maximum intensity projections of taxol-treated dorsal forewing scale phenotypes from injected and fixed *Plutella xylostella* pupal wings 24hrs after injection. Wings visualized for F-actin (X, yellow) and acetylated α-tubulin (X", blue), and their overlap (X'). Scale bars measure 25μm. Distal scale tips oriented to the right.

Fig. II.12 ACME dissociation of diverse pupal wings at scale growth stages



Examples of cell mixes from 72HAP *Junonia coenia* (A,A') hindwings and a 48HAP *Greta oto* (B) forewing dissociated at early scale growth stages using a modified ACME protocol. Cell bodies (red) and nuclei (blue) are labeled with propidium iodide and DAPI, respectively. Dissociated cells remain intact, retaining early scale projections (A'). Scale bar in (A) measures 50µm. (A') and (B) were viewed using a ZEISS Observer Z.1 compound microscope with 40X and 20X objectives, respectively.

Fig. II.13 Alternative hypotheses for the control of scale shape by Cut



Distinct hypotheses for the role of Cut in generating diverse scale types (A) fate change or (B) altered morphogenesis:

(A) The presence (+) or absence (-) of Cut splits developing scales into either cylindrical (right branch) or flattened (left branch) scale types, where differentiating factors yet to be identified (X1&2) would further subdivide the cylindrical scale into different classes, like sensory or cilia.

(B) Scales progress through development according to distinct phases punctuated by processes (arrows) representing transcriptional regimes of cytoskeleton-binding proteins or other factors that determine shifts in growth pattern and cell shape. In the case of cylindrical scales, Cut represses the transcriptional transition from growth process #1, maintaining it later throughout development. This inhibition could be specific to the scale flattening process (#2), in which case process #3 can still be activated, or complete, in which case process #1 continues until the scale is fully grown.

Chapter III: Convergent evolution of scales and color patterning in a genus of caddisfly (Trichoptera: Leptoceridae: *Nectopsyche*)

III.1 Introduction

Despite the diversity of scale forms, scales of major types are usually restricted in a clade-specific fashion. In a similar vein, although scale types recur widely throughout arthropods, variation in scaled and scaleless species are usually drawn along general lines, with very few genera being variable for the presence or absence of scales. One such example are *Strumigenys* ants where the 'squamiform setae' adorning the head capsules of some species are so strongly scalelike as to look like clamshells and in others they are long and piliform (Bolton 2000). Dissecting the genetic influences underlying morphologies is expedited when there is variation between species or populations with which to craft comparative analyses. A system where there have been major roadblocks in this regard are lepidopteran scales. All lepidopteran scales are derived from a bauplan consisting of a flat lower lamina connected by trabeculae to an adwing upper lamina adorned with micro-rib patterned ridges which define two borders of windows into the lumen otherwise delineated by cross ribs connecting the ridges (Thayer & Patel 2023). As all lepidopteran scales have this suite of characteristics, or are derivations thereof, it is not possible to investigate the origin of any of these traits through comparative analyses.

Scales are thought to have arisen once at the base of the Amphiesmenoptera, the superorder containing both the extant orders Lepidoptera and Trichoptera, and then retained in the former and lost in the latter, being replaced with an eponymous "hairy" pattern of bristles/wing hairs instead of a scales (Wang *et al*. 2022). Despite this, scales have evolved again multiple times convergently within the Trichoptera, as determined parsimoniously by their sporadic distribution across genera. Most of these instances consist of sex-specific differences in patches of macrochaetae differentiated into widened androconial scales, but some involve cases of the entire pelage becoming scale-like, either constitutively, like with the genus *Pseudoleptocerus*, or in a sexually dimorphic manner, as in the remarkable *Protoptila diabolita* (Huxley & Barnard 1988; Robertson & Holzenthal 2008). A rare example of a genus within which the presence of constitutive scales is variable between species, meaning some species completely lack scales and others are fully lepidopterous, is the New World *Nectopsyche*, or miller caddisflies (Holzenthal *et al*. 2015).

Nectopsyche are a diverse group within the Nearctic and Neotropical regions composing 103 described species (59 in actuality, discounting the 2017 "phenomic" treatise whereby 44 species were defined solely by extremely subtle and likely inconsistent differences in male genitalia) known for their extremely colorful forewing patterns, especially in relation to other Trichoptera which have a reputation for being quite drab (Trichoptera World Checklist 2023; Oláh & Oláh 2017). These wing patterns are composed of specific motifs, the most striking of which is the jumping spider mimic pattern which has been recorded in many different insects, even outside the Amphiesmenoptera (pictured in Fig.III.1) (Hill 2022; Greene *et al*. 1987; Zolnerowich 1992). Understanding of the evolution of these wing patterns and macrochaete diversity is complicated

by the lack of phylogenetic attention given to this genus, despite the incredibly diligent taxonomic attention it has received (Holzenthal & Calor 2017). Neotropical species in the *gemma* group were the last section of the genus revised, with no species-level molecular phylogeny having been produced (Holzenthal 1995).

This study aims to rectify the systematic neglect of the genus *Nectopsyche* and determine the set of relationships defining the major clades of the group. Likewise, an effort is made to characterize the diversity of macrochaete types and their evolutionary transitions using morphological analysis of their ultrastructure in relation to determined phylogenetic groupings. Ecological function is also briefly tested with a short behavioral analysis in relation to wing pattern evolution and presumed antipredator defense. In another foray, the morphological diversity is contextualized mechanistically with a brief developmental analysis to determine the cell biological actors which may be prefiguring certain macrochaete elements. Lastly, recognizing the wealth of opportunity provided by the unique variation in morphological traits relating to wing pattern and macrochaete ultrastructure within this genus, efforts are outlined to develop rearing protocols for the lab culture of a selected species, *N. exquisita*. As part of this, a genome is constructed for this species to expedite future studies investigating the specification of wing pattern elements and macrochaete classes.

III.2 Methods

III.2.1 Phylogenetics and genome sequencing

29 *Nectopsyche* and five outgroup species belonging to other genera within Leptoceridae were loaned from the University of Minnesota Insect Collection and Brigham Young University from the personal collections of Drs. Ralph Holzenthal and Paul Frandsen. Each specimen was photographed before and after removing and storing the wings for macrochaete sampling. One individual from each species was then ground up and DNA extracted using the *Quick*-DNA Miniprep Plus kit (Zymo Research). An anchored hybrid enrichment (AHE) probe set was designed based on Trichoptera transcriptomes produced by the 1KITE initiative using scripts from a similar AHE study on flower fly phylogeny (Misof *et al*. 2014; Young *et al*. 2016). Regions of the transcriptomes that had conserved sequences with variable flanking regions were selected to construct the biotinylated nucleotide probes which bind to DNA and can then be differentially extracted with magnetic streptavidin beads (Lemmon *et al*. 2012). Sequencing was outsourced to RAPiD Genomics, LLC (Gainesville, FL) and conducted using an Illumina HiSeq2000 platform with 2x150 cycles.

Sequencing data were processed on the Office of Research Computing high-performance cluster at Brigham Young University (Provo, UT) using a slightly altered version of a pipeline from Dr. Jesse Breinholt (Breinholt *et al*. 2017; Rázuri-Gonzales 2020). In summary, paired-end read adaptors were removed using TrimGalore, with remaining sequences fewer than 20bp pruned from the dataset (Krueger *et al*. 2019). Trimmed reads had gaps removed and corresponding amino acid sequences were aligned in MAFFT v.7.455 using an L-INS-I algorithm and orthology ran using Orthrograph software with sequences from *Danaus plexippus*, *Bombyx mori*, and

Tribolium castaneum input for comparison (Katoh *et al*. 2002; Petersen *et al* 2017). Codons were aligned using a custom PAL2NAL script, modified from v.14.1 (Suyama *et al*. 2006). Phylogenetically uninformative sites with random sequence similarity were removed using a custom version of ALISCORE, with sliding window set to the default of 4 (Misof & Misof 2010; Kück *et al*. 2010). ALINUC-generated nucleotide alignments based on the amino acid sequences were processed with ALICUT to remove any segments within these alignments that were determined to be randomly similar. FASconCAT 1.0 was run to combine processed nucleotide and amino acid alignments into a supermatrix, removing any loci recovered in fewer than 40% of sampled species (Kück & Meusemann 2010). Phylogenetic trees were constructed using IQTREE v.1.6.12 (Nguyen 2015). To determine the best nucleotide substitution model and partitioning scheme, loci were first merged using TESTMERGEONLY; ModelFinder was then run while allowing variable partition rates (Kalyaanamoorthy *et al*. 2017). 25 independent maximum likelihood tree searches were run using the determined optimal partitioning scheme from ModelFinder, with 1000 bootstrap replicates each. The tree with the best maximum-likelihood score was then chosen and visualized using FigTree v.1.4.4 (FigTree 2023).

A genome from a -80ºC flash-frozen *N. exquisita* pupa was also constructed. DNA extraction and sequencing were carried out by the Brigham Young University DNA Sequencing Center (Provo, UT) using the protocol outlined in (Ríos-Touma *et al*. 2022). To summarize here, high molecular weight was extracted using the Quick-DNA HMW MagBead Kit (Zymo, DS1710-03) and visualized before sequencing via gel electrophoresis to verify sample quality. The SMRTbell Express Template Prep Kit 2.0 (PacBio, 100-938-900) was used to make a library, with DNA sheared to 15kb beforehand according to the PacBio low input protocol recommendations for HiFi sequencing. The library was cleaned up using AMPure beads and then ran on a single 30-h movie 8M SMRT cell in CCS mode on a Sequel II system (PacBio). Raw HiFi reads were generated using the pbccs tool in pbbioconda and a k-mer count histogram created using KMC v.3.1.1 through GenomeScope2.0 to get summary statistics and an estimate of heterozygosity (Pacific BioSciences 2021: Kokot *et al*. 2017; Ranallo-Benividez *et al*. 2020). HiFi reads were assembled into contigs using Hifiasm v0.13-r307 with aggressive duplicate purging (Cheng *et al*. 2021). Afterwards, validation for capture of expected insect orthologs was run with BUSCO v.4.1.4 using the Insectaob10 and Endopterygota odb10 core sets (Manni *et al*. 2021; Kriventseva *et al*. 2021). Gene annotation is still ongoing.

This chapter would not have been possible without collaborating with researchers from the University of Minnesota and Brigham Young University. DNA from the *Nectopsyche* spp. for phylogenetics were extracted by Ernesto Rázuri-Gonzales and Paul Frandsen. Library construction and sequencing were outsourced to RAPiD Genomics, LLC (Gainesville, FL). The phylogeny was produced under the supervision of Paul Frandsen during a visit to Brigham Young University with remote help from Jesse Breinholt in the final stages of model selection. DNA extraction and sequencing for the genome were outsourced to the Brigham Young University DNA Sequencing Center from a specimen I collected in northern Virginia. Initial genome assembly pipelines were primarily run by Paul Frandsen on the Office of Research

Computing high-performance cluster at Brigham Young University, who also walked me through the quality check software.

III.2.2 Collection, rearing, and behavior

N. exquisita were collected in northern Virginia, USA during mid-May and early-June. Adults were attracted to a UV-blacklight trap consisting of either a white bedsheet or paint bucket and kept in wide-mouth Mason jars without the lids and the opening covered by cut panty hose sealed tight with the ring. To collect eggs, saturated fabric towels were placed on the mesh-covered jar opening to increase the relative humidity of the chambers. Within about an hour, eggs could be collected from the moist walls of the jar with a soft paintbrush. Adults were given a sugar water-dampened paper towel at the bottom of the jars for nutrition, ensuring the towel is damp and not soaking or saturated, as the caddis will get stuck if it is too wet.

Larvae and pupae were picked off of rocks along the river bottom. Use of a D-net to sweep the vegetation was occasionally fruitful in catching some swimming individuals, but hand-picking was most productive. Collected egg masses and wild-caught larvae and pupae were raised in glass bowls filled with spring water (Poland Spring). Larvae and hatchlings were given germinated wheatgrass kernels with the roots and cotyledons intact. Hatchlings additionally had 50-325 mesh sandblasting beads (Kramer Industries, Piscataway, NJ) added to serve as substrate for casemaking. The water was changed every two days to prevent the build-up of protists and an aerator constantly ran to ensure proper oxygenation.

N. gemmoides were sampled at the Smithsonian Tropical Research Institute in Gamboa, Panama in mid-October and late January from streams within the Parque Nacional Soberanía along Pipeline Road using a blacklight for adults at night and a D-net to capture larvae. To assess the function of salticid mimic wing patterns, wild-caught adults were paired in overturned Petri dishes with size-matched salticid jumping spiders found nearby the stream where the caddis were collected, and their interaction recorded with a Samsung Galaxy S8. Salticids were starved for twelve hours before introducing *N. gemmoides* into the arena and no caddisfly was used more than twice for encounters with a spider. Likewise, spiders were only used five non-consecutive times before they were retired for that day of caddis exposures. Observational notes were taken on their interaction.

III.2.3I Immunohistochemical staining

Pupal wings were dissected and stained for cytoskeletal polymers exactly as described in Chapter II. Briefly, wings were dissected in 4ºC PIPES:EGTA:$MgSO_4$ (PEM) buffer and fixed for 30min in 3.2%PEM-PFA before washing 3x5min, 3x30min in PBS and staining with 1:200 WGA conjugated to Alexa Fluor 488 in PBS for 1hr at room temperature. Afterwards, wings were washed 2x5min with PBS then permeabilized with 0.1% Triton X-100 PBS (PT) 1x5min, 2x30min washes. Blocking was carried out for 30min in 5% normal donkey serum in PT before the antibody solution was added. To visualize microtubules and actin, 1:200 phalloidin conjugated to Alexa Fluor 555 (Invitrogen, A34055) and 1:500 mouse α-acetylated α-tubulin primary antibody (6-11 B-1) conjugated to Alexa Fluor 647 (Santa Cruz Biotechnology, sc23950

AF647) were used alongside 1µL 1µg/mL DAPI and kept overnight at 4°C. The next day, wings were thoroughly washed for 3x5min and 3x30min with PT before storing in 70% glycerol at 4°C.

III.2.4 Imaging

Confocal images of the macrochaete actin and microtubule cytoskeletons were acquired using a ZEISS LSM880 Airyscan inverted confocal with the following ZEISS objectives: 5x Fluar 0.25NA, Plan-Apochromat 10X 0.45NA, Plan-Apochromat 20X 0.8NA, and C-Apochromat 40X 1.2NA water and using the Airyscan detector with appropriate filter sets for Alexa Fluors 488, 555, and 647. To capture high-resolution color photographs of macrochaetae, either individual scales were picked off wings with tungsten needles or whole wings were mounted onto a glass slide and imaged with a Keyence VHX7000 using reflected light on the glass stage insert to give wings and scales a neutral grey background. Tungsten needles were also used to carefully dissect portions of the scale in the case of structurally colored *N. gemmoides* in order to observe the effect on iridescence. Scanning electron microscopy was performed by mounting scales or whole wings onto carbon tape and sputter coating with platinum at a coverage of ~3nm. Sputter coated samples were then imaged on a ZEISS Supra 40VP using the secondary electron (SE2) detector and an electron high tension (EHT) at 3.0kV.

III.3 Results

III.3.1 Salticid mimic wing patterns are polyphyletically distributed

A maximum likelihood phylogeny was successfully constructed for 29 species of *Nectopsyche* using 249 anchored hybrid enrichment loci with 100% bootstrap support on almost all branches and high values (>75%) within *Nectopsyche* otherwise (Fig.III.1). A monophyletic *Nectopsyche* was recovered, with an immediate sister group of the clade uniting *Brachysetodes*, *Amphoropsyche*, and *Neoarthripsodes* leptocerids (Fig.III.1). *Parasetodes bankeri* was found to be most closely related to *Adicella longiramosa* in the tree's outermost-branching clade. *Nectopsyche* itself was divided into ~9 major clades in a graded pattern, with *N. acutiloba* being the basalmost branching, followed by a major clade of *N. brunneofascia*, *N. aureovittata*, *N. muhni*, and *N. maculipennis* + *N. aureofasciata*, *N. gemmoides*, *N. argentata*, and *N. gemma* (*gemma* clade). This is followed by *N. ortizi* and *N. pantosticta* (*ortizi* clade) branching, and then another composed of *N. fuscomaculata*, *N. navasi*, *N. bruchi*, *N. muelleri*, and *N. separata* (*separata* clade). Thereafter, *N. splendida*, *N. cubana*, *N. punctata*, and *N. flavofasciata* (*punctata* clade) split. Two sets of species pairs then branch in series: first, *N. exquisita* and *N. candida* (*exquisita* clade), then *N. stigmatica* and *N. dorsalis* (*stigmatica* clade). Finally, *N. spiloma* and *N. dorsalis* (*dorsalis* clade) and *N. tavara*, *N. albida*, and *N. diarina* (*albida* clade) are found to be sister.

Wing patterns throughout the genus are diverse, with species ranging from iridescent silver and orange-yellow amongst the *gemma* clade, to fawns and whites in the later-branching ones, with even some iridescent green popping up in the appropriately named tiny *N. splendida* of the *punctata* clade (Sup.III.1). Overlaying wing pattern occurrences over this series of relationships, it is obvious that salticid mimic wing patterns occur in disconnected patches around the

phylogeny, at least four times in the *gemma* branch of the *gemma* clade, *N. ortizi* of the *ortizi* clade, *N. flavofasciata* of the *punctata* clade, and *N. exquisita* of the *exquisita* clade (Fig.III.1).

The metalmark moth genus *Brenthia* displays similar, but differently arranged jumping spider-resembling patterns created by the overlap between both sets of fore- and hindwings. In the presence of salticids, these moths have been recorded to engage in behaviors that include facing the spider and raising both sets of wings to center their display, to which the would-be assailant spider responds with territorial displays or backing off entirely, giving the moth time to fly away (Rota & Wagner 2006). This set of interactions suggests these patterns may be a predator mimicry defensive strategy. In the spirit of these experiments, head-to-head trials were conducted between *N. gemmoides* and jumping spiders. When placed in overturned Petri dishes together, some (N=4 of 15 trials) *N. gemmoides* displayed a distinctive behavior wherein they turned to show their flank to the encroaching spider and waggled their abdomen, at which point the spider either turned or back away (Sup.III.2). A more common behavior elicited upon approach (N=9) was to fly away, which in the enclosed conditions of the Petri dish resulted in knocking into the spider and startling it aback. Twice, the spider was successful in capturing and consuming the *N. gemmoides*; having fed, these spiders were removed from the trials.

III.3.2 Nectopsyche *scale architecture is mosaic for lepidopteran traits*

Along with the evolution of complex wing patterns themselves, the macrochaetae composing them have diversified within the *Nectopsyche*. Broad, flat iridescent scales adorn wing bands and patches in several *Nectopsyche* spp., especially those with jumping spider mimic wing patterns (Fig.III.2A,B). The abwing surface of *N. gemmoides* iridescent scales is composed of many tightly packed ridges and the absence of windows, in comparison to black scales of the same outline that have wider spaced ridges with circular fenestrations at regular intervals between them (Fig.III.3, black scales left, iridescent scales right). Removal of the abwing surface of iridescent scales removes the iridescent effect, with the sloughed upper lamina retaining a sheen (Fig.III.2D). *N. ortizi* also have silver iridescent scales, but these have wide spacing between ridges on their adwing surface and a completely smooth lower lamina (Fig.III.4C,C').

In contrast to fly bristles, all large trichopteran macrochaetae observed were flattened. Thus, in these structures the major axis of variation determining designation as a scale and not a "scale-hair" was width, with the former having shafts expanded into a racket shape as opposed to being slender ovoid or rectangular. Scales and scale-hairs of the same color in *Nectopsyche* therefore looked identical microstructurally, save one appearing to have been significantly widened. The eponymous covering of smaller wing hairs closer to the wing surface were all piliform, by contrast (this "understory" of projections visible in the background of Fig.III.3B'). Wing hairs are deceptively not homologous to macrochaetae as they are not socketed and instead form as multiple projections from the surface of an epithelial cell instead of being wholly formed from a single trichogen cell (Adler & Charlton 2000). This difference in size effectively creates a "forest" on the wings: a dense understory pelage of cylindrical wing hairs loomed over by the canopy of macrochaetae that define the wing pattern. The presence of at least some portion of a flat lower lamina on the adwing surface of all macrochaetae was actually found to be widespread throughout a number of trichopteran families in a two separate surveys, suggesting that

trichopteran macrochaetae have been largely mischaracterized as hair-like or cylindrical (Wagner *et al*. 1996; Stocks 2010).

Macrochaete ultrastructure was sampled throughout the different clades in the phylogeny, representing the named species of the *albida*, *exquisita*, *punctata*, and *ortizi* clades and *N. gemmoides* in the *gemma* clade plus *Parasetodes bankeri* as an outgroup representative (Sup.III.3). Macrochaetae in *P. bankeri* were very slender, with deeply corrugated smooth ridges. Between these ridges were sparse windows, with other non-perforated windows adjacent to these. The adwing surface was overall smooth with the exception of two prominent parallel ridges that ran the length of the shaft. *Nectopsyche* scales present variously with a range of adwing surface textures. *N. exquisita* and *N. albida* lower laminae resemble *P. bankeri*, each with two ridges running the shaft length, but, in contrast to *P. bankeri*, these were deeply recessed into a concavity between these ridges, making them appear much more dramatic. Between the species with racket-shaped scales, adwing surfaces presented as either smooth or with many parallel ridges that can be independent or linked by subtle crossribs into a reticulated pattern (Fig.III.4C',B',D', respectively). Abwing surfaces of non-iridescent scales had many more windows, presumably to expose pigments deposited into the lumen or lower lamina. As window number increases and adjacent windows press against each other, cross ribs are formed with a width in inverse proportion to the window area, such that the largest windows have the thinnest cross ribs. All trichopteran scales observed lacked microribs and many lacked flared processes on top of ridges, with exceptions in *N. exquisita*, where they are incredibly subtle, and *N. albida*, where they are greatly elaborated (Fig.III.4E,F).

Twice during the morphological survey of macrochaete architectures, aberrant scales were found in *N. exquisita* wings that were very obviously more scalelike (one example pictured in Fig.III.4B). These aberrations were both intermediate in color between orange and white, being a light creamsicle, and around twice the width of an orange macrochaete, but while retaining the same number of adwing ridges. Along with the increased inter-ridge distance, windows were also larger than expected for an orange macrochaete. The general outlines of the aberrations were racket-shaped like a scale, but, due to their rarity, the risk was not taken to remove them from the wings to view the adwing surface and determine if they had a smooth lower lamina, as well.

III.3.3 Nectopsyche *macrochaete growth follows similar trends to Lepidoptera*

Actin and microtubule cytoskeletons in initially projecting *N. exquisita* macrochaetae displayed the same overlapping organization as has been recorded in early moth wings (compare Fig.III.5A' with Fig.II.1A'). At the other end of the developmental process, macrochaetae were occupied by uncoupled actin and microtubules: the former arranged in rods defining the ultimate position of ridges and the latter reticulated into a diffuse network throughout the length of the shaft (Fig.III.5B).

III.3.4 Developing Nectopsyche *as an evo-devo system*

In order to expedite the study of the development and evolution of wing patterns or their constituent macrochaetae from seasonal work to feasible year-round, *Nectopsyche* must be able to be reared in lab and wing discs must be able to be dissected to follow standards set by studies

of lepidopteran color pattern (Martin & Reed 2014). Towards this first end, significant effort was expended developing rearing protocols for wild-caught *N. exquisita* larvae and eggs masses.

Although CO_2 gas exposure has been reported to have an oviposition-inducing effect on caddisfly females, this result was not able to be replicated in *N. exquisita* (Resh 1972). Instead, it was found that egg mass laying could be induced by increasing humidity in enclosure jars by draping a wet towel over their pantyhose mesh openings. Eggs were able to be retrieved with a soft paintbrush and placed into aerated spring water. Larvae hatched in batches from the outside of the egg mass in, with their developmental progress visible through a nearly totally transparent chorion. Development took around one to two weeks, depending on temperature regime and level of oxygenation, with warmer and more oxygenated embryos developing the fastest. Hatched larvae were prolific crawlers and quickly composed a makeshift first case out of the eggshell and any available debris but were easily trapped at the water interface without sufficient ballast. A number of fine commercial glitters were tried but all were too big to be manipulated by the first instar larvae. Eventually, glass beads 45-150μm in diameter were determined to be adequate for first case construction.

In the wild, larvae were found to have a strong preference for horn-leaved riverweed, *Podostemum ceratophyllum*, being found almost exclusively on this plant despite many other plants growing in the river ecosystem. Propagating of *P. ceratophyllum* was not attempted as it seemed to prefer only rocky substrates, but, in pursuit of an easier aquatic plant, larvae were challenged with Java moss, *Vesicularia dubyana*; water milfoil, *Myriophyllum* spp.; and curley-leaf pondweed, *Potamogeton crispus*. Larvae did not consume any of these plants, despite the latter two genera being listed as primary food stock for *N. albida* (Tozer *et al*. 1981). Inspired by reports of caddisfly larvae occasionally being found as pests of rice paddies, another poaceous plant, *Triticum aestivum*, was grown from seed to first sprouts as wheatgrass and given to wild-caught larvae (Balduf 1939; Hicken 1967). After an acclimation period of a day, larvae were found chewing on the wheatgrass seed kernel and base of the sprouting leaves, and older larvae consumed the roots (Fig.III.6A). This supplementation was adequate to facilitate molting and pupation in medium-sized larvae which would normally fail to pupate in the absence of additional food. Additionally, finely ground *T. aestivum* seeds were presented to newly hatched larvae and sustained them through a molt to the point when they begin decorating their case with the dorsal stick archetypical of *Nectopsyche* cases (Fig.III.6B) (Wiggins 1996). A negative effect of using wheatgrass as a food source was the tendency for cultures to experience explosions of protists if water changes were not conducted at least every other day. As a testament to how severe the blooms could get, wheatgrass media is the preferred substrate to rear *Vorticella* peritrichs in culture (Sun *et al*. 2012). Sterilizing seeds before germination with a quick bleach treatment delayed protist blooms, but an ideal solution has not yet been identified for dealing with the water fouling and larval die-offs these population explosions elicit (Lindsey *et al*. 2017).

Attempts to dissect imaginal discs from *N. exquisita* larvae were initially fruitless, with no discernable discs being present in the thoraces of medium-sized larvae collected from the wild (and therefore true instar unknown). Only in one somewhat larger larva were thickened bands of tissue that suggested some imaginal nature ever found. These were attached to lines of

sclerotized interior cuticle wall visible from the exterior of the dorsal thorax. In the prepupal stage, when larvae began to seal their cases with silk pads to ready for pupation, these thickened bands of tissue began to rapidly proliferate into imaginal discs proper, in fact being visible even through the cuticle at these stages as it began to swell in preparation for apolysis (Fig.III.7). Forewing and hindwing discs were found to have extremely different shapes even early on, with the former resembling the typical round-edged lepidopteran disc and the latter being sharply triangular. From dissecting these discs from multiple larvae across a range of different stages of the pre-pupal period, it was apparent that growth was particularly rapid and was coincident with rapid changes in shape (Fig.III.7B').

Along with animal husbandry developments, genomic resources would facilitate the investigation of gene expression and open the door for future genetic manipulations using CRISPR (Choi *et al*. 2018; Mazo-Vargas *et al*. 2017). A genome with an estimated 1Gb size and approximately 20X coverage was successfully obtained using HiFi sequencing of an *N. exquisita* pupae using low-input protocols. Genome statistics were overall favorable, with a BUSCO score of 98.7% (App.III.1). Strikingly, the estimated heterozygosity from GenomeScope was very high at 4%.

III.4 Discussion

One of the primary uses of the assembled phylogeny is assessing the taxonomic relationships that have been inferred already from morphological examination. The other genus within the tribe Nectopsychini, *Parasetodes*, was not found to be the closest branching genus to the *Nectopsyche*, supporting similar findings from a molecular phylogeny of the Leptoceridae which also found Nectopsychini to be paraphyletic (Malm & Johanson 2011) (Fig.III.1). No formal phylogeny of the *Nectopsyche* has been attempted before, with the closest approximation being informal taxonomic working groups defined by similarity between members. Such "groups" may be interpreted as rough analogs for perceived clades but in practice are purely a shorthand without systematic implications. For instance, careful morphological analysis of genitalic characters had already indicated the paraphyly of the taxonomic '*gemma* group' due to subtle differences in the morphology of the male terminalia of *N. ortizi* and this study indeed recovered that this species did not fall within the *gemma* clade (Holzenthal 1995). In light of this new evidence that it belongs to a separate clade, there are several pattern elements of *N. ortizi* that can be observed *post hoc* that distinguish it from the *gemma* group, including radiating lines of silver scales along the veins at the base of the dorsal forewing and an additional black "eyespot" along the posterior wing edge (Fig.III.1).

Viewed in relation to that of the moths and butterflies, the arrangement and distribution of macrochaete ultrastructure within *Nectopsyche* is quite curious. Outside the presence of microribs, all of the major features defining lepidopteran scales are present within the *Nectopsyche* as a whole, but this completeness breaks down when singling in on the characteristics of single species (Fig.III.8). That is, the distribution of scale traits is heterogeneous, or, more accurately, mosaic, with respect to the lepidopteran bauplan elements

within the *Nectopsyche*. In the evolutionary sense, it suggests that evolution of these traits is rather straightforward as it takes place within the divergence time of species pairs. In the developmental sense, it suggests these elements are under independent genetic control as they pop in and out of the phylogeny. And in the practical sense, it actually facilitates comparative analysis between species to dissect the specification of distinct microstructural elements, since there is now variation between these species where there was previously none in the whole of Lepidoptera. To further expand on this point, as sensory organ precursor cell derived structures are all homologous, the scale characteristics composing them can also be thought of as being shared and linked through a common evolutionary toolkit of the SOP lineage. In this way, a ridge in a lepidopteran and trichopteran scale can be thought of as parallel outputs of ancestral processes, wherein even if knowing how the microstructures in one are patterned does not tell exactly how the other is patterned, they are expected to share mechanistic links that can expedite the process in the absence of naturally occurring variation between a more closely related species.

Overall, the vestiture of Trichoptera was found to differ than initially expected. While indeed covered with cylindrical wing hairs, the "non-scale" macrochaetae were not cylindrical in cross-section, but instead flat like scales, just with a much reduced, but still distinct, lower lamina. The question of scale evolution in *Nectopsyche*, therefore, becomes one of broadening the trichogen projection instead of reorganizing its actin to produce circumferential ridges and a cylindrical profile. This variation allows a different process of scale morphogenesis to be considered, as the factors differentiating scale-hairs from scales would affect the extent to which flattening occurs as opposed to just its presence or absence. As a semantic note, the term scale-hairs is adapted from "scale-like hairs", becoming hyphenated to avoid suggesting their affinity with non-homologous wing hairs but retaining historical contingency with the literature (Whalley 1986).

This potential for molecular inquiry of species pairs with and without certain microstructures is bolstered by that fact that it seems trichopteran cytoskeletal organization has similar correlations to macrochaete shape as has been recorded in the Lepidoptera (Chapter II). Initially projecting scales have highly coordinated, overlapping filamentous actin and acetylated α-tubulin cytoskeletal networks, which is uncoupled in later scale growth, just as in flattened lepidopteran macrochaete phenotypes (Fig.III.5). Along with emphasizing the generality of these cytoskeleton-macrochaete structure relationships, they provide a foundation with which to interpret processes and hypothesize aspects of morphogenesis in trichopteran scales, as they share a common "amphiesmenopteran logic". The added occurrence of spontaneous aberrations within *N. exquisita* that appear more scale-like dangles the possibility that a simple switch activating a scale program exists. In this context, these scale-like phenotypes seem to be the result of errant activation of a "widening" program in normally narrow, rectangular pigmented macrochaetae, as the number of ridges remains the same. In this sense, the muted color may suggest that the original identity of these scale-like aberrations were orange scales, wherein pigment production was not increased along with widening, yielding the less intense color as the same amount of pigment was now occupying a larger volume.

Scales, despite being single cells, have a tremendous impact on organismal fitness as they compose the color patterns governing vision-modulated interspecific interactions. For

Nectopsyche, the fact that spiders retreated after *N. gemmoides* displayed their wings is a promising glimmer of evidence for some anti-predator function in these wing patterns, as this was the same response seen during spider challenges in behavior trials with *Brenthia* moths (Rota & Wagner 2006). This trait should be further explored in more regimented and quantitative behavioral trials, taking into account evolutionary scaling relationships. Jumping spider mimicry was found to be sensitive to relative size of spider vs prey in the cases of both *Brenthia* and *Ceratitis* fruit flies, and therefore the observed intermittent salticid mimic pattern evolution may be linked to shifts in wing size, as this trait is very variable within *Nectopsyche* (Sup.III.1) (Rota & Wagner 2006; Hasson 1995). A scenario could exist whereby at small or intermediate sizes, this wing pattern evolves for predator mimicry functions, but as other factors drive miniaturization or larger body sizes, these defenses become less effective and the patterns are either not maintained and vanish with genetic drift or additional selective forces apply and drive the evolution of alternate wing motifs, perhaps for the evolution of crypsis.

Iridescence was found to be a common trait in species of *Nectopsyche*, often but not exclusively co-occurring with salticid mimic color patterns. Because those wing patten traits are polyphyletically distributed, this presents opposing hypotheses of, like the wing pattern itself, structural coloration either having been evolved multiple times or retained from an ancestral program that has been continually redeployed throughout *Nectopsyche*. Scale microstructure can help to distinguish these possibilities: if the iridescence mechanisms within instances of salticid mimic patterns are the same, an ancestral program might be maintained or been switched back to in these species. If, by contrast, the iridescence mechanisms are different, then it is more likely that these wing patterns are convergent. Iridescence of *N. gemmoides* likely results from an undulatory sawtooth thin film reflector on the upper lamina, as ridge number is much increased relative to pigmented scales, windows into the lumen are absent, and dissection of the upper lamina abrogated iridescent color (Vukusic *et al*. 2008). *N. ortizi* iridescent scales lack the sawtooth array of ridges on the upper laminal surface and instead show increased distances between ridges and a completely smooth lower lamina, as has been recorded for silver lepidopteran scales that produce their color using lower lamina thin film interference (Ren *et al*. 2020). This difference in iridescence mechanism and corresponding scale microstructure between *N. gemmoides* and *N. ortizi* points towards these iridescent scales, and perhaps the wing patterns forming them, being convergent. This raises the question of why these functional convergences of reflective scales against a field of orange-yellow would evolve throughout this group. One hypothesis is that this combination maximizes contrast in the wing pattern using already at-hand genetic programs like ommochrome and melanin pigment synthesis and the modulation of cuticle secretion in layers of developing scales. Jumping spiders are known to be sensitive to pattern differences related to contrast, more so than the constituent colors of patterns themselves, and so this combination of bright reflected light on a colored background may increase the effectiveness of these wing patterns as a predator mimicry defense (Girard *et al*. 2018; Taylor & McGraw 2013).

Nectopsyche wings are unusually patterned overall for the Trichoptera; and this peculiarity is such to the extent that most species are described based on differences in wing pattern alone—

making them unique amongst Trichoptera in that they are preserved pinned rather than in the usual storage in ethanol which would remove the wing covering making it nearly impossible to identify them from their mostly homogenous genitalia. How such distinctive wings patterns have evolved seemingly *de novo* is as much a question as how once the wing pattern arose it diversified throughout the genus. Developing wings have intrinsic pattern signals that dictate and pattern morphogenesis and it is possible that *Nectopsyche* wing pattern elements are acting on a pre-existing signaling. Most wing color known from Trichoptera derives from pigments being deposited directly into the wing membrane, as exemplified by the charismatic phryganeid genus *Eubasilissa* (Kuranishi & Hsu 2019). It is known from *Drosophila* that similar genes that pattern the colors of scale cells in butterfly are evolutionary deployed to create melanization patterns on dipteran wings (Koshikawa 2020). Perhaps, in this case, developing sensory organ precursor cells have evolved to become receptive to these ancestral wing-membrane pigmenting signals to lay the groundwork of a "first" wing patterning. The differentiation of macrochaetae themselves, once it has taken place, may also be an evolutionary catalyst for the evolution of further wing patterning by providing "grist for the mill" of a combination of either drift or natural selection to tinker with in distribution and frequency. To explain the much greater diversity of wing patterns in *Nectopsyche*, it can be first noted that other leptocerids form simple wing patterns formed from a few dots or stripes from the wing pelage (Ames 2008). This might suggest that the evolution of *Nectopsyche* wing patterns was evolutionarily predisposed from this foundation, wherein the evolution of a novel macrochaete class could have hypothetically kick-started pattern formation and subsequent selection into specific motifs. Supporting this, perhaps, is the fact that one of the other instances of a caddisfly with a complicated pattern of coloration is another leptocerid in the Afrotropical genus *Pseudoleptocerus*—which sports even more scale type classes than *Nectopsyche* (Huxley & Barnard 1988). Regardless, study of *Nectopsyche* and related genera lacking a notable wing pattern could reveal how color patterning initially evolves.

The investigation of wing pattern evolution in Lepidoptera has been inextricably linked to viewing patterns of gene expression within the larval imaginal discs and, with this in mind, dissections of wing discs from *Nectopsyche* were attempted. Up to this point, the Trichoptera have been reported as having imaginal discs present throughout the larval stage, just as in the Lepidoptera; yet, this condition was not found for *N. exquisita*, which only began to proliferate imaginal tissue right before pupation (Marshall 1913; Ling 1934). Despite what may be interpreted from the imaginal disc literature which is heavily biased toward cyclorrhaphan flies, the strategy of delaying growth until the prepupal stage is actually quite common in insects and is highly variable even within orders (Niitsu *et al*. 2021). In addition to *Nectopsyche*, beetles like *Tribolium castaneum* and the Scolytinae, most Neuroptera, *Chaoborus* midges, sawflies, and scorpionflies all lack proliferated imaginal disc tissue until the last instar (Shah *et al*. 2011; Powell 1904; Sehnal *et al*. 1996; Weissman 1866; Švácha1992). The distribution of this characteristic, in fact, suggests it is the ancestral condition for holometabolous larvae and that sequestered and early developing imaginal discs are the derived character along this spectrum (Niitsu *et al*. 2021). In Trichoptera then, in light of this new evidence, *Nectopsyche* may have gone back to a more ancestral mode of imaginal disc growth or the sampled earlier-branching families had each evolved pre-prepupal stage imaginal discs independently, though augmented

taxa sampling would increase the ability to assign polarity to this character in the caddisflies (Thomas *et al*. 2020). Ironically, this condition makes *N. exquisita* slightly more viable as a system to study wing pattern evolution, as, even though they currently lack a closed life cycle in the laboratory, the access to earlier instars conferred by such a protocol would be unnecessary as they would lack any significant prepatterned imaginal tissue to dissect. Therefore, collecting larvae as they pupate from the wild actually saves resources, even if it means tissue can only be collected a few times annually.

Still, rearing *N. exquisita*, and ideally another member of the genus like *N. gemmoides* which presents with stereotypical scales, would ultimately prove more convenient for gene knockdown and yearlong investigations. In this sphere, much progress has been made in this report, with only the hurdle of preventing water fouling in the introduction of the wheatgrass diet preventing the continued growth of larvae hatched from induced egg masses in culture. Switching to a system that automatically changes water would mechanically solve this problem, albeit running the risk of trapping early instars in filters. Chemical treatments that discourage protist growth would likely be the more ideal introductions. Once a lab-line of *Nectopsyche* spp. is established, genes known to prefigure lepidopteran wing color distribution like *optix* can be used as jumping-off points to investigate possible convergent roles in the specification of *Nectopsyche* pattern elements, probing for expression and targeted mutagenesis by using the assembled genome to create primers (Thulluru *et al*. 2022). Whichever genes found to be responsible for specifying color patterns can then be assessed with respect to the ostensibly convergently evolved salticid mimic patterns, as has been done for similar cases of convergence in *Heliconius* (Joron *et al*. 2006).

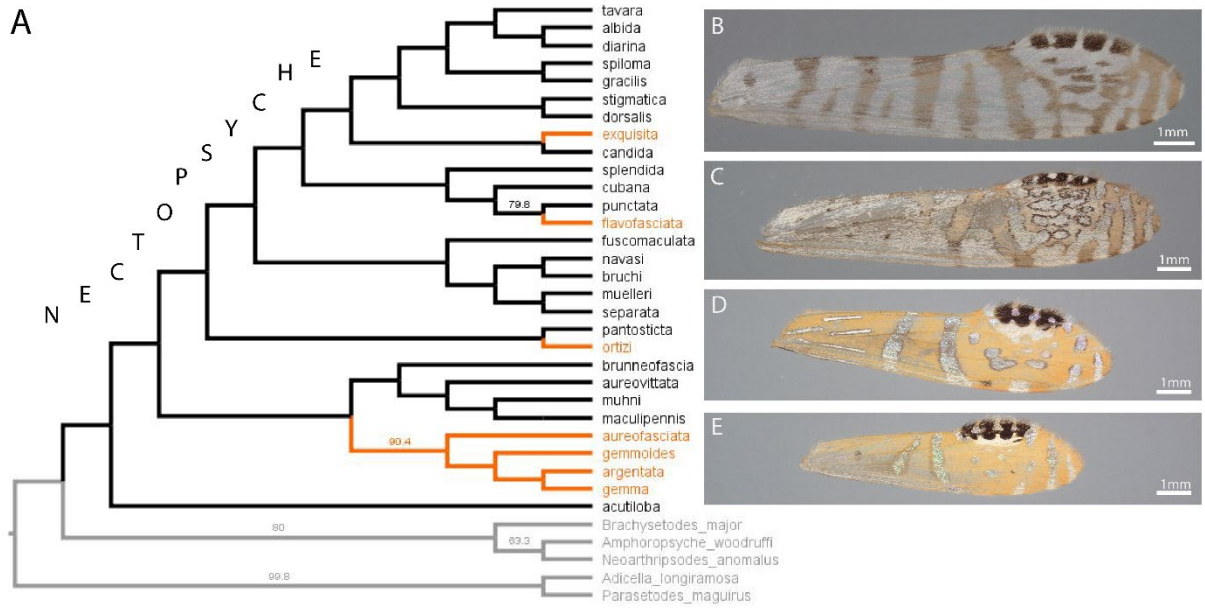
The development of these resources itself could also hold the potential to open new questions and yield new leads into the specification of color patterns. Sequencing of the *N. exquisita* genome revealed it to have a high heterozygosity value, which could potentially suggest that the sampled population was experiencing introgression from another population. An interesting candidate for this introgression is the sister species to *N. exquisita*, *N. candida*. *N. candida* and *N. exquisita* populations have considerable overlap and surveying wing phenotypes across their ranges from public insect image repositories like bugguide.net and iNaturalist shows the presence of several individuals that are difficult to place as *N. candida*, which has an off-white wing with black spots like an ermine coat, or *N. exquisita* which has a salticid mimic pattern, because either the salticid mimic pattern is extremely hazy or the ermine coat pattern has distal elements that resemble the salticid mimic pattern. In light of the high heterozygosity recorded, these individuals could be interpreted as potential hybrids of the two species with a mix of the parental wing patterns. Validation of whether these two species are interfertile could, excitingly, provide a system to uncover the specification of wing-pattern elements through QTL-mapping of siblings between parents of non-admixed populations of each species (Thayer *et al*. 2020).

III.5 Conclusions

Their high diversity in wing pattern and range of macrochaete architectures that appear to recapitulate common trends in scale evolution, especially in the context of microstructural elements, make *Nectopsyche* a promising system to study the evolution of wing patterns and

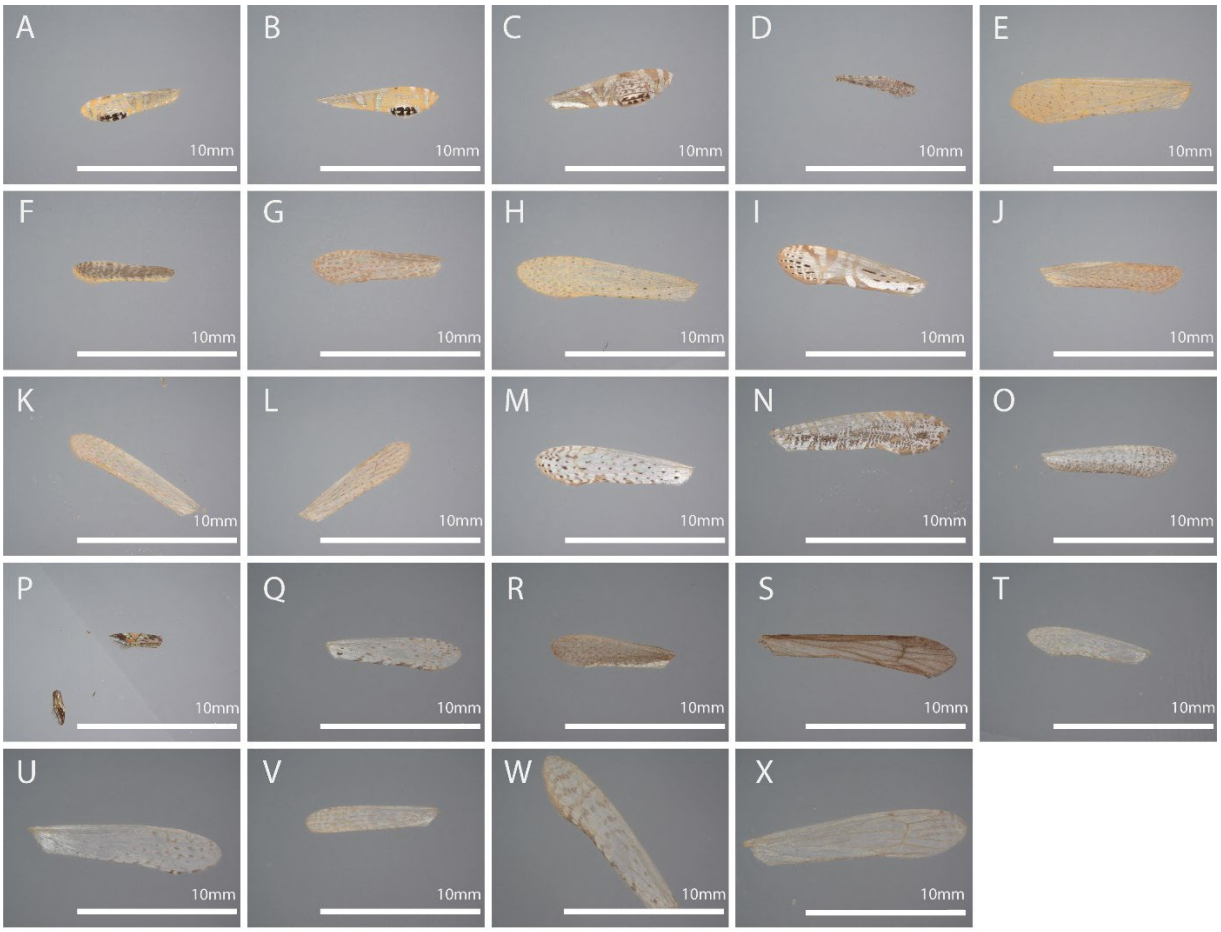
scale traits at the level of a clade—an approach recently being championed to give a better view of evolutionary processes than selecting single species to serve as "models" (Donoghue & Edwards 2019). Using species-pair comparisons variable for macrochaete traits archetypical of lepidopteran scales has the potential to uncover the molecular and cellular specification of these features. Through overlaying this information with the evolutionary processes by which these traits are scattered throughout the phylogeny to confer different morphologies making up wing pattern motifs and significant fitness effects in predator-prey interactions, this system stands out for its ability to transcend our understanding of the influence changes in cytoskeletal organization have on organismal fitness. Moving forward, looking for correlates between ultrastructure and other traits like wing size or opsin repertoires could reveal ecological interactions in this sphere. Furthermore, collecting additional phenotypic data from and including other clades within *Nectopsyche* would give a more solid foundation for hypotheses of evolutionary transitions in wing pattern composition and macrochaete morphogenesis.

Fig. III.1 *Nectopsyche* phylogeny and the evolution of salticid mimic wing patterns



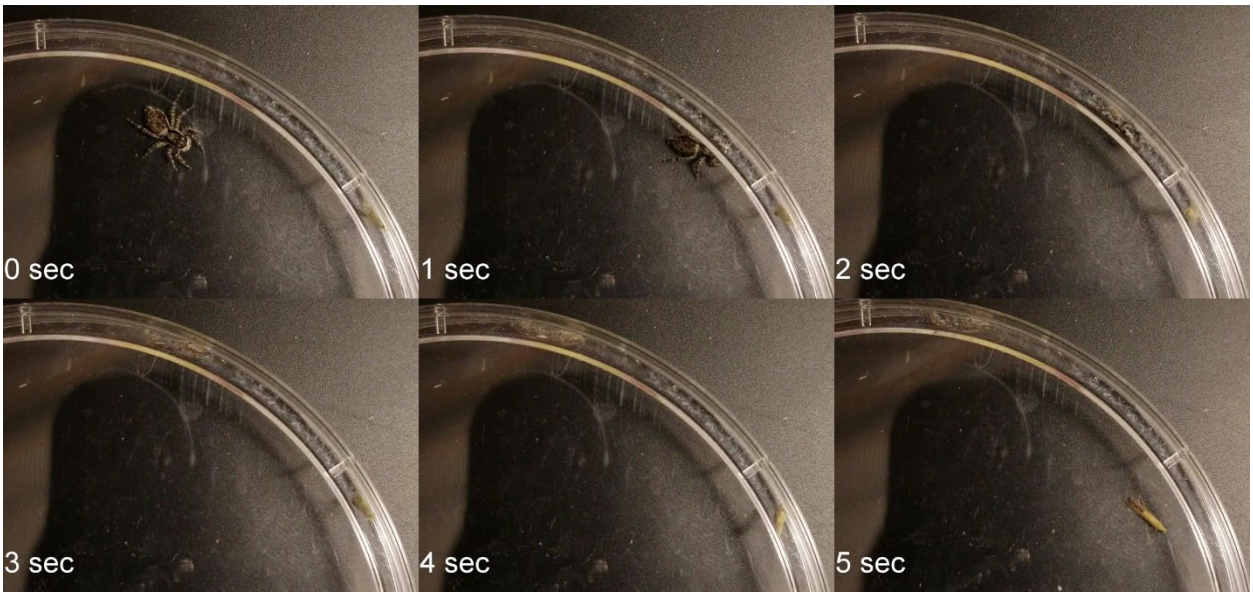
(A) Maximum likelihood phylogeny of 29 *Nectopsyche* miller caddisfly species and 5 confamilial leptocerid species as outgroups (grey clades). Species with a salticid mimic wing pattern are colored in orange including *N. exquisita* (B), *N. flavofasciata* (C), *N. ortizi* (D), and the *gemma* clade (*N. gemmoides* pictured, (E)). Only bootstrap values of less than 100 are shown on branches. Forewings in (B-E) arranged with posterior margin up to emphasize the pattern when the wings are held at rest alongside the body. Scale bars equal to 1mm.

Sup. III.1 Representative forewings of *Nectopsyche* spp. in the phylogeny



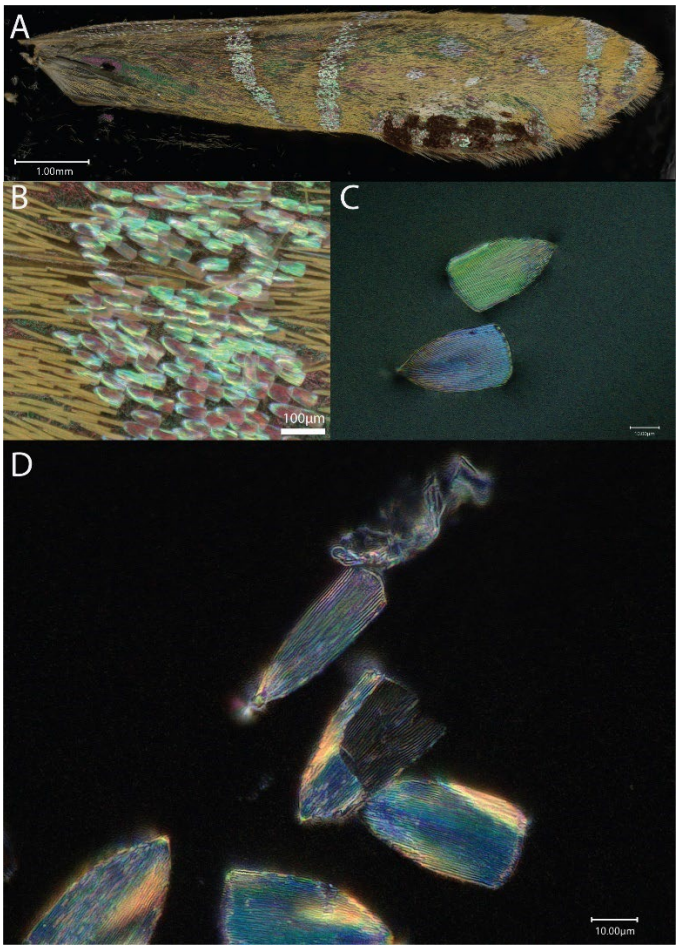
Color photographs of male wings from the species sampled in the phylogeny not already pictured in Fig.III.1: *Nectopsyche gemma* (A), *N. argentata* (B), *N. aureofasciata* (C), *N. maculipennis* (D), *N. muhni* (E), *N. aureovittata* (F), *N. brumeofascia* (G), *N. pantosticta* (H), *N. separata* (I), *N. muelleri* (J), *N. bruchi* (K), *N. navasi* (L), *N. fuscomaculata* (M), *N. punctata* (N), *N. cubana* (O), *N. spendida* (P), N. *candida* (Q), *N. dorsalis* (R), *N. stigmatica* (S), *N. gracilis* (T), *N. spiloma* (U), *N. tavara* (V), *N. diarina* (W), and *N. albida* (X). *N. acutiloba* not pictured. Scale bars measure 10mm.

Sup. III.2 *Nectopsyche* salticid mimic wing patterns may have anti-predator function



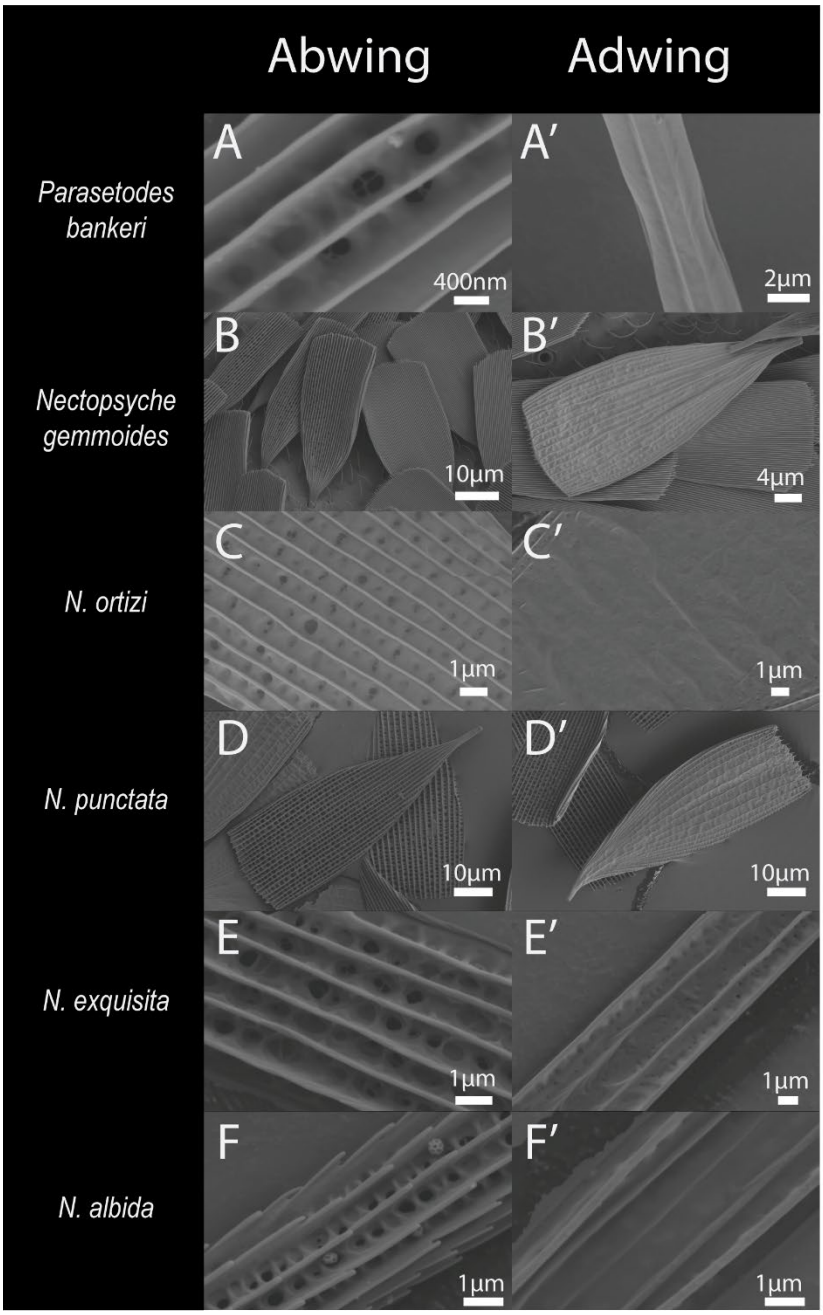
Montage of movie capturing the approach and retreat of a salticid jumping spider investigating an *N. gemmoides* in an artificial arena. The possible anti-predator display occurs at 2sec, with the caddisfly orienting its side toward the spider and waggling its wings. To this, the spider backs away. Montage created from sampling each second of the movie.

Fig. III.2 Scales and structural coloration in *Nectopsyche*



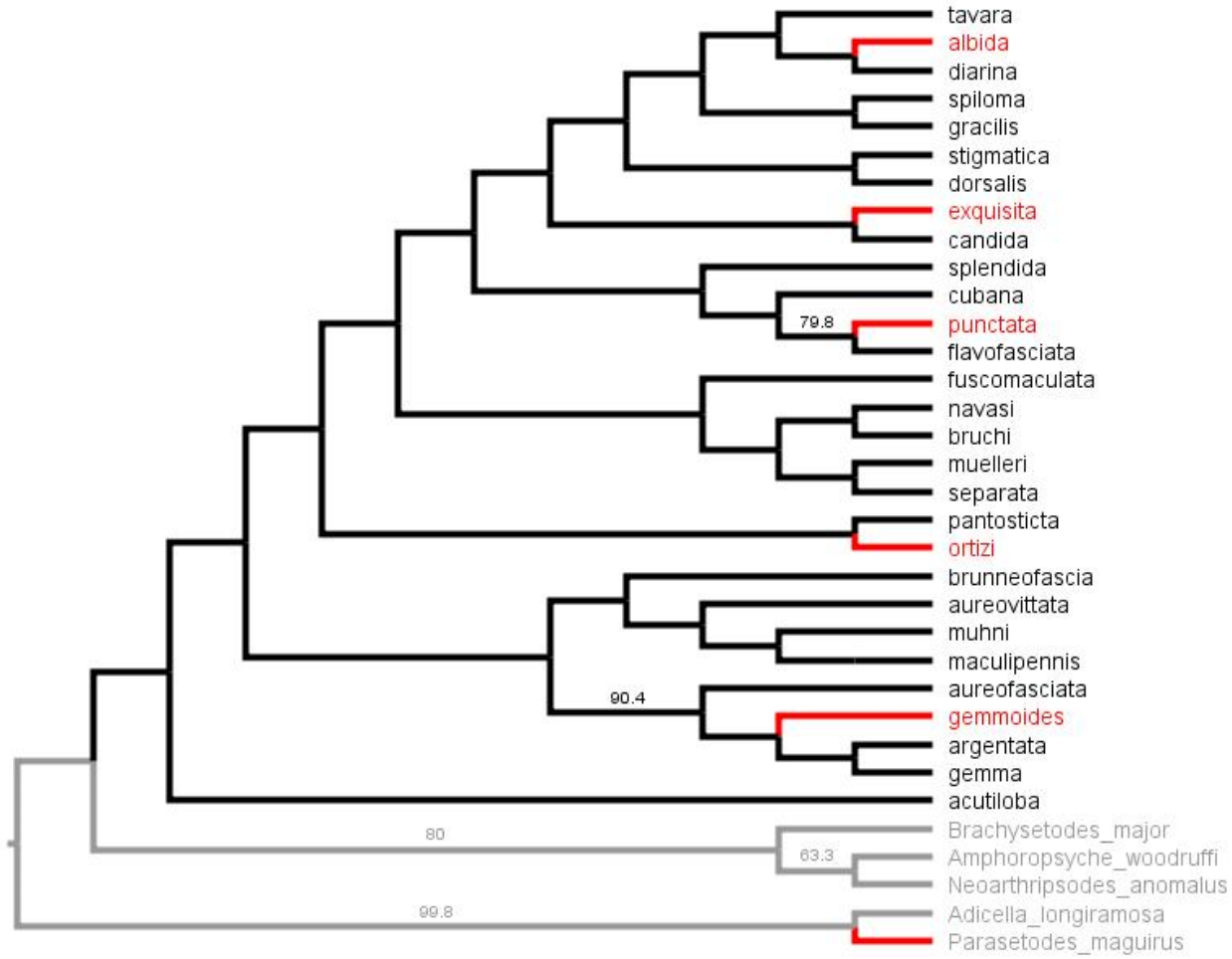
High resolution color photographs of a *N. gemmoides* wing at 400X (A-whole wing; B-scale area), 2500X (C-Scale close-ups), and 2000X (D-Scale close-ups). In (D), part of the upper lamina of one of the iridescent scales has been dissected off. Scale bars measure 1mm (A), 100µm (B), and 10µm (C&D).

Fig. III.3 *Nectopsyche* scales are mosaic for Lepidopteran scale traits



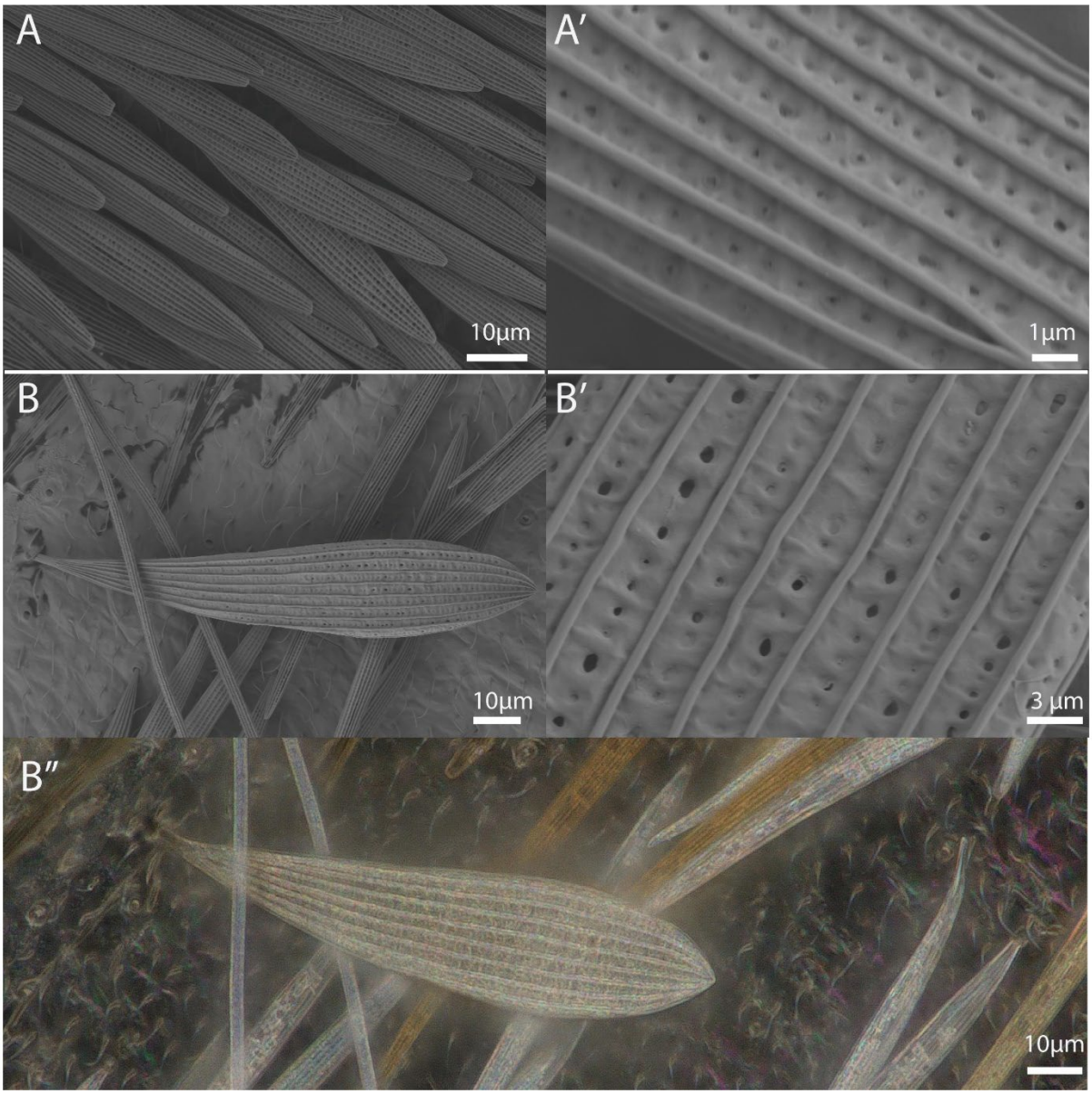
SEM micrographs of abwing (X) and adwing (X') scale structures throughout an outgroup leptocerid and five *Nectopsyche* spp: *Parasetodes bankeri* (A), *N. gemmoides* (B), *N. ortizi* (C), *N. punctata* (D), *N. exquisita* (E), and *N. albida* (F). Scale bars measure 400nm (A), 2µm (A'), 10µm (B,D,&D'), and 1µm (C',E,E',F,F'). Buckminsterfullerene-like spheres in (F) are brochosomes produced by leafhoppers, which commonly adhere to pinned specimens, and not part of the scale anatomy (Wu *et al*. 2023).

Sup. III.3 Phylogenetic context for SEM species sampling in Fig.III.3



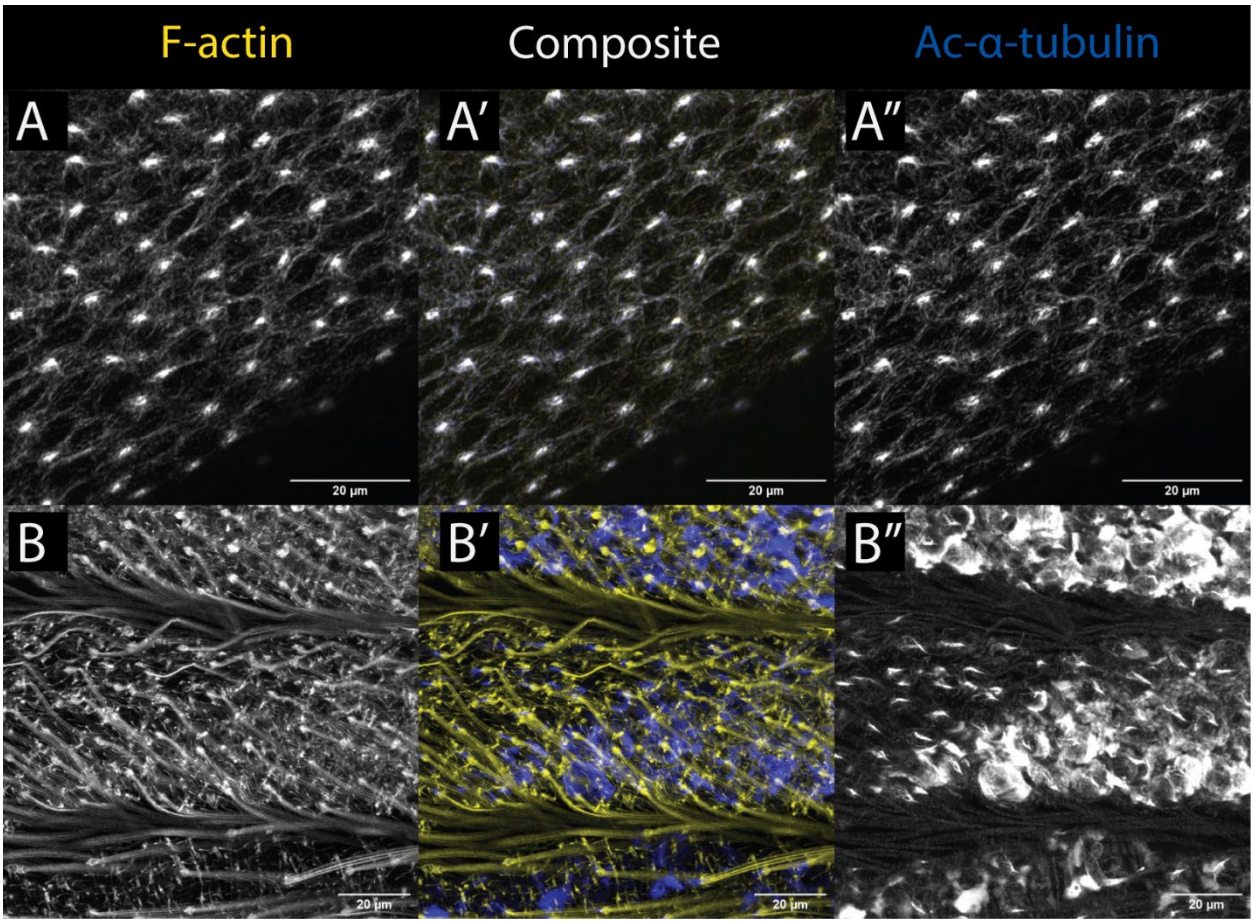
Distribution of species sampled for scale microstructures in Fig.III.3 (labeled red): *Parasetodes bankeri*, *N. gemmoides*, *N. ortizi*, *N. punctata*, *N. exquisita*, and *N. albida*.

Fig. III.4 An aberrant scale-like morphology from *N. exquisita*



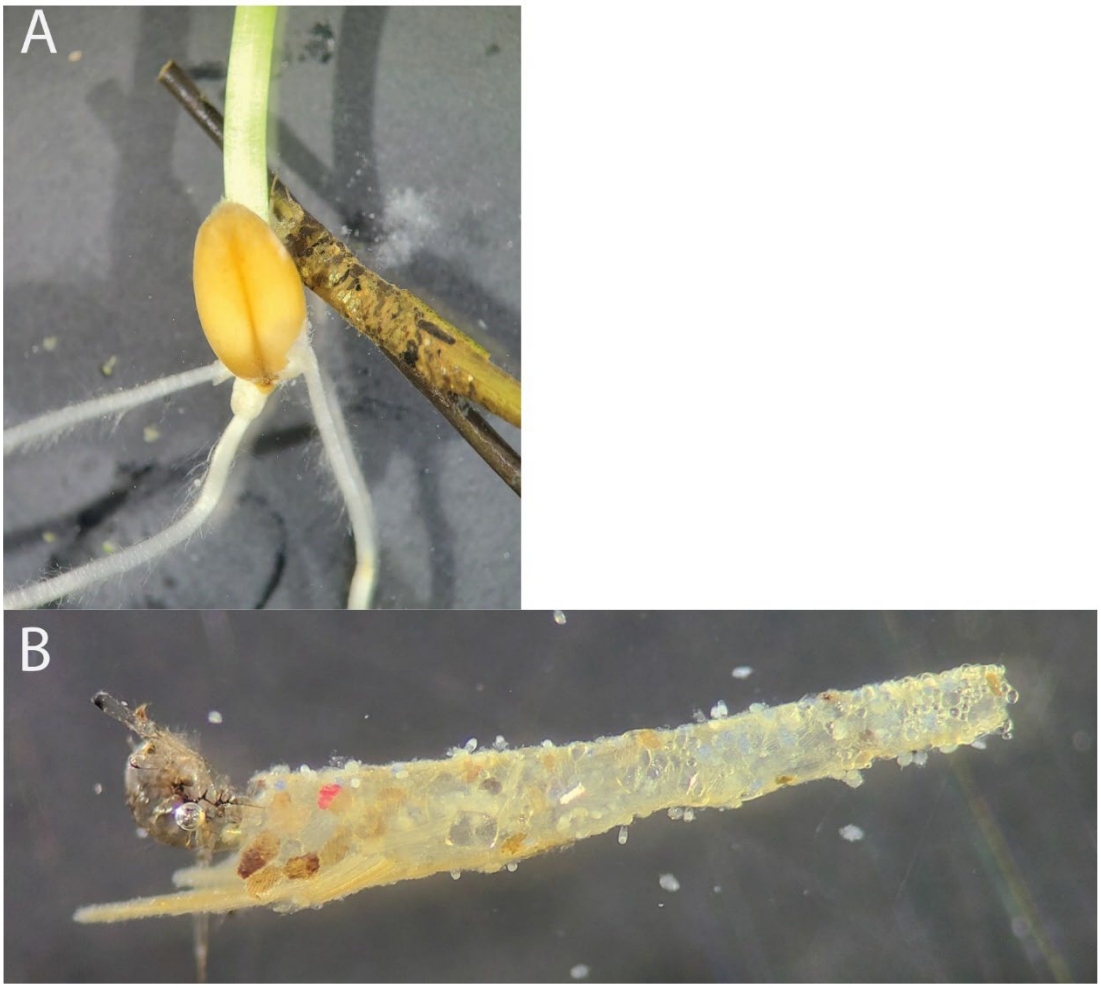
An example of typical pigmented macrochaetae on *N. exquisita* wings (A,A') and a lone scale-like aberration viewed under SEM (B,B') and with a high-resolution color camera (B"). (A') and (B') represent close-ups of the abwing surface. Scale bars measure 10µm (A,B,B"), 1µm (A'), and 3µm (B').

Fig. III.5 *N. exquisita* cytoskeletal organization in developing macrochaetae



Visualization of the cytoskeletal organization of filamentous actin (X-yellow), acetylated α-tubulin (X"-blue), and their overlap (X') in *N. exquisita* macrochaetae just starting to project (A) and almost fully extended (B). Macrochaetae positioned with distal to the right in (B). Scale bars measure 20µm.

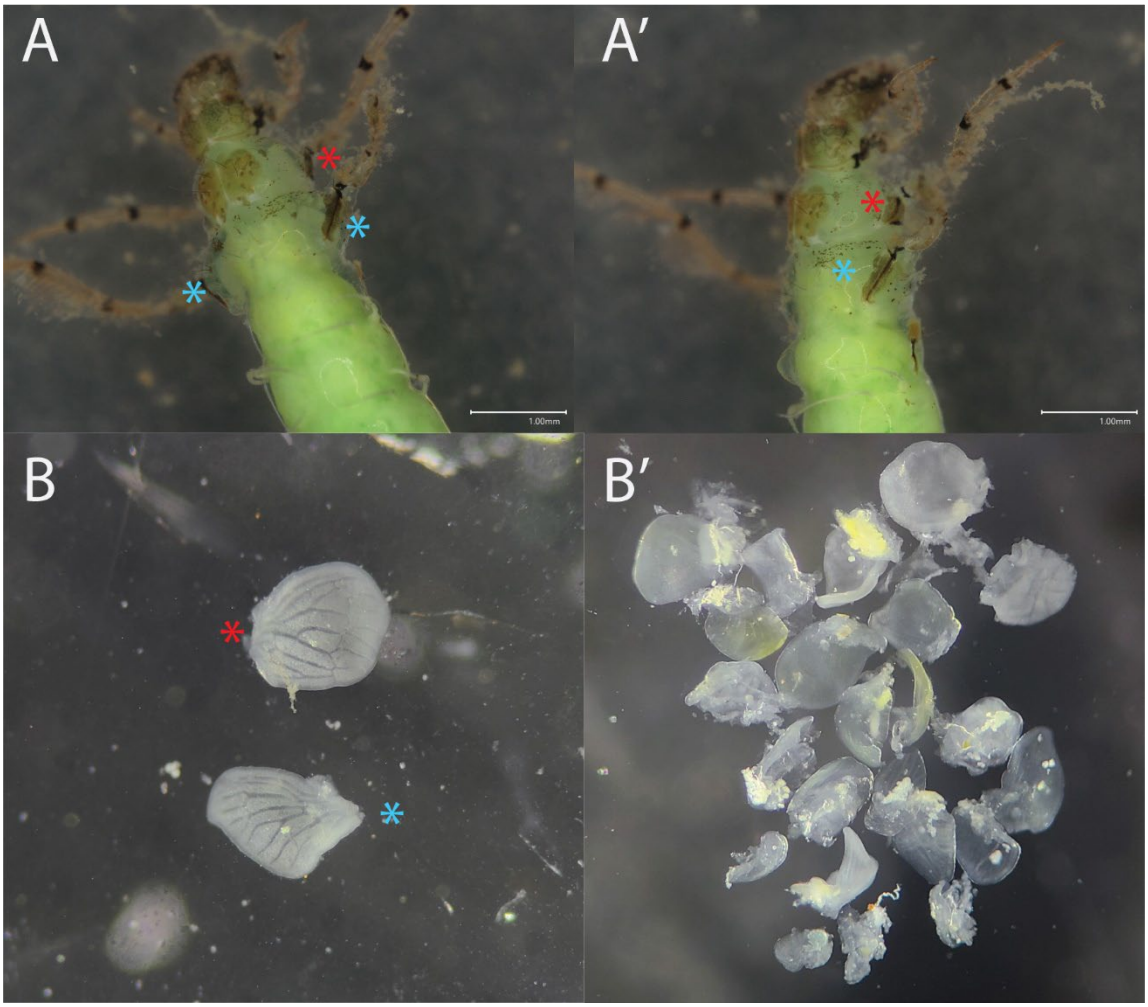
Fig. III.6 *N. exquisita* larvae can develop on wheatgrass in culture



(A)-Photographs of a *N. exquisita* larvae consuming a wheat kernel and sprout, indicated by the white crumbs on the glass dish bottom near dorsal side of larva.

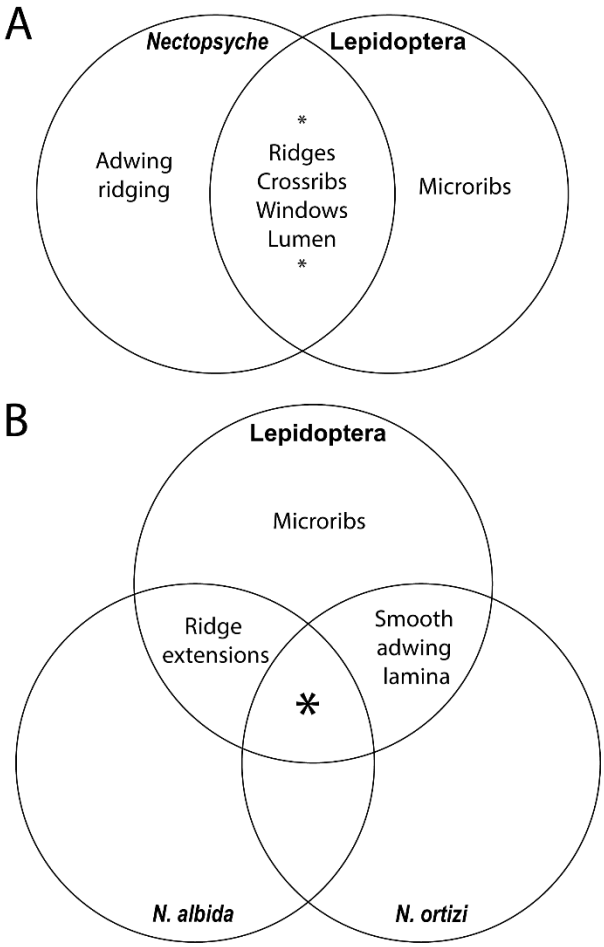
(B)- Empty case and head capsule of a wheatgrass-fed larva hatched from eggs collected in the lab and developed to ~second instar. Note that the artifical case made of glass beads features the characteristic *Nectopsyche* inclusion of a long dorsally attached stick.

Fig. III.7 *N. exquisita* imaginal discs do not proliferate until the prepupal stage



Dorsal (A) and lateral (A') view of a mid-prepupal stage *N. exquisita* and dissected imaginal discs (B,B'). Discs in (B) dissected from the larva in (A&A'). Forewing and hindwing discs marked by red and cyan asterisks, respectively. Scales bars in (A) and (A') measure 1mm.

Fig. III.8 *Nectopsyche* as a clade to dissect the evolution of scale traits



Venn diagrams populated with examples of the mosaic characteristics of scale feature evolution in *Nectopsyche*.

A- With little exception, *Nectopsyche* as a genus has macrochaetae with the elements that can be found in the lepidopteran scale bauplan (asterisks).

B- However, different *Nectopsyche* spp. have different combinations of these lepidopteran-like features.

Conclusion

Through the work of this document, I hope to have convinced any reader on the immense utility of studying the epithelial appendages of insect exoskeletons. To end, I present musings aiming to contextualize this study into wider biological thought and construct a working model to act as a scaffold for future investigations. First, I note the unique characteristics of insect scales that make them so useful at parsing the evolution of morphogenesis, then I use what is known from macrochaete heterogeneity to link how it may be possible to predict evolutionary process just from morphological comparisons. I finish with a note on how the methods I have developed and outlined in this document may aid in closing a lacuna between what I argue to be opposed sub- and supracellular levels of focus in modern evolutionary-developmental biology.

Scales as a nexus for questions of biological hierarchy

Biological organization can be thought of as a nested series of levels corresponding to differing degrees of interaction. Proteins make up macromolecules, which are encoded by genetic material inside cells which organize into tissues that make up organs. Organs coordinate to comprise the organism which interacts with other similar individuals to assemble into a population. Diverse populations interact forming an ecosystem, and the collection of ecosystems on earth compose the biosphere. The hierarchical nature of this organization has traditionally stratified biological fields into a limited set of layers, for example ecology as looking solely at how populations interact in an ecosystem and how ecosystems crosstalk, but increasingly studies have aimed to reach across these tiers with the thought that in doing so a more comprehensive assessment of reality can be gleaned. In this way, scientists studying particular genes involved in assembling limbs could, for example, interpret mutations through biomechanical assays that assess appendage function at the organ level and then change the environmental parameters to determine fitness in an ecological context which may have been important during periods of evolutionary transitions. Structural biologists interested in the biophysics of light transduction within the molecular makeup of chlorophyll can similarly investigate differential efficiencies between wavelengths of light and link this to primary productivity and carbon cycling with the atmosphere. Both of these examples illustrate the power in being able to connect evidence from one field of study with the broader perspectives of another to achieve synergy. But while such investigations work well within one species at a single point in its lifetime, some aspects can become unwieldy if not impossible when attempting to incorporate evolutionary trajectories.

Comparison forms the basis of biological empiricism. Scientific investigation is possible because like things can be grouped, isolated, and perturbed to sus cause and effect. As structures change throughout evolutionary time, relationships between features become hypotheses known as homologies (Wake *et al*. 2011). In essence, structures are homologous when they are believed to have been inherited from the same common ancestor. In practice, homology is more akin to a process itself, with context determining its efficacy as a term: homology must be expressed as a

relationship. For an illuminating example, the wings of bats and birds are not homologous as wings because their common ancestor did not have wings—their wings have convergently evolved in the clades leading to modern birds and bats; however, these structures are homologous as forelimbs—their common ancestor did possess a set of forelimbs from which both bat and bird wings, despite being modified heavily along the way, have evolved. In this manner, features can be homologous with respect to one level of biological organization, but not another (Abouheif 1997). If a gene is recruited across disparate cellular contexts to achieve a similar key role, these instances can be thought of as homology for gene function, despite the structures themselves that they are patterning arising from entirely different cell populations and areas of the body. Such a process is called 'gene co-option' and has recently faced skepticism as to how common such instances really are, but similar scenarios can arise on a practical, if not biological, level when a feature has been displaced positionally in relation to its closest relatives through catastrophic mutational shift or, more commonly, when intermediate lineages have gone extinct without leaving behind morphological remnants, making comparisons impossible (Bruce & Patel 2022).

Essentially, crafting and framing comparisons is necessary when interpreting phenomena with respect to evolutionary processes. For this reason, due to the inability to establish clear links between structures, evolutionary-developmental studies of multicellular eukaryotes have split into basically two types: 1) those that look at protein structure and function, consequences of mutational changes to these proteins, and subsequent divergence of genes encoding these proteins and their phenotypes in the cells or organs of a single species and 2) investigations correlating differences, be it spatial, temporal, or quantitative, in organization of gene presence, expression, and regulation to differences in features between variable traits in a polymorphic population or across branches of life. Both approaches address development in an evolutionary light, the first by looking at how building blocks can be tinkered to have downstream effects on the construction of tissues and the second by watching the tape of life as it has played out in biodiversity to produce endless forms. What is missing, however, is a link between these types of studies, which can nearly be impossible as it is not usually straightforward to look at a population of cells and coherently link one in particular to another in a cell population of a different species and continue this in sequence across thousands of years of evolutionary change.

In specific instances, conserved populations of cells can be compared across species through a shared and stereotyped organization. A fruitful system in this case is the secreted integumentary appendages of amniote skin: reptile scales, bird feathers, and mammalian hairs (Oster & Alberch 1982). Each of these seemingly disparate secreted products can be evolutionary linked through the similar method by which their secretory apparatus differentiates. For each appendage, a placode patterns an overlying follicle of multiple cell types that coordinate their growth to excrete keratin in the shape of the corresponding outgrowth in a manner akin to a 3D printer (Di-Poï & Milinkovitch 2016; Kondo *et al*. 2018; Duverger & Morasso 2009). Although considerably less studied, polychaete worm chaetae similarly form through export of a polymer, in this case chitin, into an ectodermal invagination by a secretory cell—'chaetoblast' (Tilic & Bartolomaeus 2016). Similar to amniote skin appendages, these bristles have been elaborated

into diverse forms throughout annelid lineages (Hausen 2005). Though even in these cases consisting of relatively few—on the order of tens of—cells patterning a structure, direct links between specific individual cells between one species and another is obfuscated by lineage specializations. And so, while the gap between more cell-focused mechanistic studies and broader evolutionary surveys of diversity can be filled at a broad level in these cases by classifying cell populations or distinct signaling centers as units unto themselves, this approach sacrifices the types of mechanistic insight that can be garnered by mutagenesis screens of individual cells.

In more exceptional scenarios, single eukaryotic cells actually assume the morphology of the structure itself instead of merely secreting it as a product. Since these structures are themselves conserved across lineages, it is very easy to identify like elements and ask questions regarding the evolution of morphogenesis. A particularly interesting developing model that fits these criteria are cnidarian cnidocytes, specialized stinging cells used in defense and feeding (Babonis & Martindale 2014). Cnidocytes have diversified across cnidarians with altered morphologies corresponding to different prey species (Fautin 2009). Additionally, some cnidocytes have lost stinging capability and instead play a role in adhering substrate to the body or entrapping food like flypaper (Mariscal *et al*. 1977; Raikova 1990). This radiation of morphologies in one cell type that can be traced from one species to the next across an entire phylum makes for an especially powerful system with which to explore the molecular and genetic basis of morphogenesis; so far, expansion of minicollagen genes has been hypothesized to serve a key role in constructing different varieties of these stinging cells (Khalturin *et al*. 2019).

Plant trichomes are another instance where the evolution of single cells can be traced across lineages. Comprising an exceptional diversity and sharing a function of protecting plants from abiotic and biotic stressors, trichomes can be unicellular or multicellular and variously associate with glands that lie on or below the extending process (Simpson 2010). Development of unicellular trichomes is exceptionally well-characterized genetically since these are the type adorning the tissues of the model plant *Arabidopsis thaliana* and the gene function of the key players has already been functionally tested across diverse species since removing trichomes is a desirable trait in plant breeding when aiming to make crops more palatable (Uhrig & Hülskamp 2010; Pattanaik *et al*. 2014; Shvachko *et al*. 2020). Although the variation in cell number throughout morphotypes and across lineages calls for caution when making comparisons between more distantly related clades, focusing within families and genera and thoroughly sampling species facilitates making clear associations between structures (Nogueira *et al*. 2013; Chopra *et al*. 2019; Watts & Kariyat 2021).

Because they too are secreted by a single cell during development, arthropod macrochaetae likewise fit into this special class of structure. This particularity has been recognized by evolutionary anatomists early on and highlighted for its usefulness in generating evolutionary comparisons (Henke 1953). Subsequent researchers classified the cellular apparatus composing individual arthropod integumentary sense organs, *e.g* sensilla, photoreceptors, and chordotonal organs, into a special category referred to as ‘organules’ (Lawrence 1966; Merritt 2006). Due to their similarity in ontogeny and arrangement, I vouch for a generalization of the organule

concept from its arthropod-specific scope to an umbrella term describing instances where specialized structures in multicellular eukaryotes are composed of either: 1) a single cell or 2) a very small population of cells descending from a single precursor and together forming a functional unit. This redefinition aims to highlight potential core principles that may link the evolutionary complement of organules across distantly related lineages and call attention to their usefulness in elucidating key features of cellular apparatuses. Already through reviewing the literature it is exciting to see hints of such commonalities, such as when comparing the development of trichomes and bristles with respect to endoreplication: *Arabidopsis* unicellular trichomes of higher ploidy are bigger and sport more branches just as scales formed from trichogen cells with higher concentrations of DNA inside their nucleus grow to a larger overall size (Zi-Yu *et al*. 2012; Cho & Nijhout 2013).

Despite originating from just one cell, just like how cnidocytes aid in prey capture and trichomes offer defense from herbivores, macrochaetae, too, can have a tremendous impact on organismal fitness (Winterton 2009). Analogous to the coverings of mammals, birds, and reptiles, the epidermal structures adorning arthropod cuticle contribute to coloration—itself a fundamental component of assortative mating, anti-predator displays, camouflage, mimicry, and countless other visual phenomena—but often neglected are the parallel thermoregulatory contributions. The incorporation of melanin pigments in scales can shorten the time taken to reach critical flying temperatures under cold stress conditions, and multivoltine butterflies exhibit seasonal polyphenism taking advantage of this property with short-day morphs being darker (Kingsolver & Wiernasz 1991). Likewise, brilliant silver hairs on the bodies of desert ants act as perfect reflectors of solar radiation, lowering effective heat conductance into the body (Shi *et al*. 2015). Beyond the incorporation of pigments, the microstructures of these projections contribute significantly to the ability to retain and dispel heat. Just as with avian down or canine undercoats, the bulk of pelage over cuticle can trap air to serve as an insulator (Church 1960). To this point, finite element modeling of different scale architectures across a wing provides a relief map of particular morphologies which aid in retention of heat along key areas of the wing necessary for pheromone production, independent of pigment value (Krishna *et al*. 2020; Tsai *et al*. 2020). This down further contributes to acoustic dampening which may aid in avoiding bat predation (Shen *et al.* 2018; Neil *et al*. 2020).

In a separate context, such macrochaete packing geometries precisely space epithelial organules of aquatic insects to hold air bubbles against breathing spiracles in order to create a ‘plastron’ or diving bell or along tarsi to leverage surface tension for locomotion on top of a water body (Flynn & Bush 2008; Finet *et al*. 2018). Apart from gripping the waves of a lake or stream, specialized bumps and brushes have convergently evolved on the distal tips of setae in soil-dwelling basicerotine and stegomyrmecine ants and psammophilous *Sicarius* and *Homalonychus* spiders for the ostensive purposes of coating the body in loose particles for the purposes of camouflage and deflecting intense ultraviolet radiation (Hölldobler & Wilson 1986; Duncan *et al*. 2007). More commonly, arthropods have evolved anti-wetting structures along their macrochaete surfaces to prevent the build-up of water and to aid in escape from droplets or the surface of liquids. Rain-sized droplets impacting butterfly wings shatter into smaller spheres as a result of

shockwave-like ripples formed upon impact; similarly, the architecture of psychodid fly scales encourages balling and easy release of water that may accumulate in their wet habitats upon light mechanical stimulation (Kim *et al*. 2020; Speirs *et al*. 2020). Not restricted to liquid-state fluids, specialized epithelial projections along the wing surface detect vibrations of air molecules in flight while a separate subclass along the wing margins contributes to the elimination of turbulent eddies that would lower the efficiency of flight (Ai *et al*. 2009; Slegers *et al*. 2017; Engels *et al*. 2021). These same long, fringed setae on the wing edge further assist the separation of the wings in cases of extreme miniaturization where insects must deal with low Reynolds number ("swimming in syrup") air dynamics: lowering drag in the initiation of flight for amoebae-sized mymarid wasps (Jones *et al*. 2016). Albeit not comprehensive, this list details broadly how macrochaetae mediate interactions between arthropods and aspects of their habitats, be it electromagnetic radiation in the forms of visible light and heat, environmental substrates where they burrow and crawl, or fluids in which they skate, swim, or fly (Watson *et al*. 2017).

When wanting to take advantage of these single-cell structural systems that span the gap between organismal function and cellular levels of biological organization, study system selection is not just informed from conceptual foundations but must also consider feasibility. With respect to these considerations, I make the argument that arthropod integumentary organules are the superior model to ask evolutionary morphogenetic questions since they leverage pre-existing genetic tools with the highest-recorded diversity of species which, generally, have short lifespans and achievable rearing conditions. Benefitting from proximity to *Drosophila melanogaster*, there is already a foundational hypothesis of the genetic control and specification of organule complexes, as well as how these factors impact morphogenesis and chaetotaxy—the distribution of macrochaete arrays on the exoskeletal surface (Simpson & Marcellini 2006). Additionally, techniques to produce transgenics can be ported from flies with reasonable success and ease (Thulluru *et al*. 2022; Heu *et al*. 2020). Insects also already have genomes assembled for 600+ species across 20 orders and their classification and morphological description have centuries of careful anatomical study (Hotaling 2021; Linné 1758; Snodgrass 1935; Wigglesworth 1950). The macrochaetae themselves are furthermore atypically feature-rich for individual cells, a fact that can be appreciated by looking at the almost architectural design of a butterfly scale, which allows for perturbations to be scored more specifically (Prakash *et al*. 2022).

By contrast, cnidarian husbandry is quite difficult and requires specialized marine water systems to upkeep pelagic species that is not achievable in all lab facilities, while their breeding and lifestyles also require conditions and care that are not well-understood (Howe *et al*. 2012; Ballesteros *et al*. 2022). Plus, their phylogenetic distance from other animals and molecular workhorses makes techniques less transferable and "plug-and-play". Plants have a number of genetic advantages in that tissue transformation can be easier than in metazoans, along with that establishing mutant lines is significantly easier with grafting and cloning, but practically can be hard to manage given longer growing times and large space requirements (Belide *et al*. 2011). Trichomes themselves vary widely in overall outline but are generally covered in a smooth cuticle that lacks features that may make implicating specific molecular actors or scoring subtle phenotypes more difficult (Kaur *et al*. 2023). Amniote integument suffers the worst of all worlds

wherein animal husbandry is very space-intensive and demanding and the alternative tissue culture very labor-intensive and costly, while still generation times are particularly long and genetic manipulation techniques either limited or nonexistent. Polychaetes approach the convenience of arthropod models in space demands but are less diverse and have dramatic spawning life cycles that make keeping colonies comparatively more complicated (Kuehn *et al*. 2019). The macrochaete system arguably excels against even evolutionary developmental protistology, a field which bypasses the issue of finding like cell populations between species by studying how intricately patterned and bizarre cytoskeletal structures are organized across organisms which, by definition, are composed of single cells. Despite also being eukaryotes, tools developed in traditional plant, fungi, and animal models notoriously do not translate reliably to protists, stalling progress. As a most egregious example, phalloidin staining does not label actin microfilaments without considerable troubleshooting in the most commonly used protist models and so outlining actin distribution becomes an onerous undertaking (Craig *et al*. 2019). Other unicellular systems like bacteria and fungi are infinitely more genetically modifiable than even flies, but their morphological diversity is often lampooned as ranging from spherical to slightly oblong. While these jests are not perhaps fair or accurate, the most striking morphologies typically occur in species deemed unculturable (Kysela *et al*. 2016).

While diversity of structures is a massive boon to the arthropod organule study system, it also in some ways makes finding a focus challenging. For this reason, I propose that homing in on arthropod macrochaetae maintains a high degree of morphological diversity to explore among species while constraining this variation to differences in morphogenesis rather than other developmental processes. Organules like chordotonal sensors or photoreceptors differ foremost in the number or arrangement of associated neurons or expressed opsin pigments whereas non-innervated macrochaetae vary principally in trichogen cell morphology. External macrochaetae are also easier to sample as they do not require tissue sectioning to get a scope of their morphology and instead can be plucked off and examined rather quickly. In narrowing the focus to macrochaetae, trends in recurrent morphologies or transitions between forms become simpler to identify. As mentioned previously, scales have evolved from bristle-like structures in arthropod lineages many times, and the multiple natural experiments of scale convergent evolution make them one of the best systems to investigate how morphogenesis may operate by specific rules or even be predictable since this recurrent phenotype is being produced in a system where genetic resources are plentiful, morphological data is well-recorded, and fitness effects can be modeled in a rather straightforward manner.

Scales, as the secreted products of single trichogen cells, stand as a nexus in the hierarchy between the sub-cellular and organismal levels of biological inquiry (Con. 1). At subcellular scales, presence of different cytoskeletal populations and binding factors can be linked genetically and biophysically to their assembly into fibers which directly impact the morphology of the final macrochaete (Overton 1966; Dinwiddie *et al*. 2014). Supracellularly, scales of particular morphologies can be localized to different areas of the body and these arrangements can have functional consequences influencing range distribution, niche, behavior, and species interactions.

A morphospace framework for the consideration of macrochaete evolution

At this point it should be obvious that the heterogeneity in macrochaete form is essential to their utility and function. Despite this, it is only becoming recently popularized to recognize that breadth in morphology when interpreting morphogenetic mechanisms. The prevailing perspectives in assessing macrochaete identity prior to the present have focused on the specification and delineation of discrete "types" at the level of the epithelial organs and their particular subclasses; for instance, a gene which regulates the shift between a chordotonal structure and a bristle, or one that specifies a red scale vs. a black one. This thesis, too, in its second chapter, is guilty of implicating a transcription factor in the differentiation of cylindrical and flattened scale morphologies. But this approach to morphology, perhaps heavily influenced by Waddington and his ever-narrowing landscapes of terminal identities, fails to capture the empirically observable biological variation in these macrochaete arrays.

Any reasonable effort to observe the assortment of epithelial projections adorning a particular axis along an organ or structure will often turn up a similar observation: gradations of form between individual macrochaetae (Con. 2). Long before molecular investigation reached the same conclusion, as early as the 18th century, such series have been documented and used as evidence to assert the homology of bristles and scales (Réaumur 1734; Kellogg 1894). But with this simple observation comes an upheaval of the current dogma: at least at some level, the biological organization of these projections no longer fits within a fixed identity model and there exist other factors influencing the lion's share of variation underlying these traits. Concomitant with this realization, I argue, is the need to alter the philosophy of approach and shift from seeking a deterministic model of the gene regulatory networks to a processual one (Dupré & Nicholson 2018). More precisely, to interpret the morphologies not as lone structures in and of themselves, but as the output of a unifying process of morphogenesis that characterizes a predisposed cell type.

Under a processual lens, variation turns outward and the study of individual ultrastructure becomes a search for morphological trends within a macrochaete field. Lepidopteran wings have served as fertile avenues for this flavor of investigation, with scale length being found to decrease from distance to the margin and overall size to correlate with the length of the wing itself (Dohrmann & Nijhout 1989; Simonsen & Kristensen 2010). Although rudimentary, these associations already can be tied into aforementioned findings relating the ploidy of cells to overall projection size. Further developments in this vein are hindered by the lack of resolution in comparing morphologies amongst a set of scales and a limit in our understanding of factors influencing the development of sensory organ precursors and their daughter trichogens. Uniting these two fronts is helped by two approaches: large-scale morphometrics and the recognition of wings as morphogenetic landscapes. To the former, careful sampling of different wing areas can glean similarities among adjacent macrochaetae, as evidenced by studies extracting scales from various patches (Day *et al*. 2019; Kohnle & Wölfling 2015). For the latter, appreciating that wings and other appendages form with and from intrinsic signaling gradients that adorning structures likewise are exposed to during development provides a starting point for investigating particular axes of variation, for instance in the consideration of morphogens (Lawrence & Struhl

1996). Still, in the current state of the field these approaches falter since asserting morphological trends is stymied by lack of sample size and the difficulty in assessing scale traits spatially instead of through lumping patches together. Similarly, despite knowing quite well the early acting molecules in establishing signaling gradients along a developing wing and being able to use the polarity of epithelial structures to roughly map the radii of influence of such candidate gradients, there exists a break between these points, wherein for early factors we do not understand what effect they may have during mid-stage pupal wing stages when scale growth initiates and the identity of the polarity-specifying factors, whether they be the same as the early signaling gradients or not, is not known (Parker & Struhl 2020; Lawrence 1966). Regardless of current stopgaps, melding these two approaches permits the assessment of morphological variation with regard to the influence of wing signaling, and the identification of molecular targets that may have more subtle impacts on shape than that which assigns scales to broader categories, *e.g.*. "cover vs ground" or a particular color. An early investigation to this effect, for instance, capitalizes on the rich knowledge of signaling within butterfly eyespots and overlays morphological measures of the constituent scale types to substantiate a ploidy hypothesis of scale type differentiation (Iwata *et al*. 2014). Moving forward, the field should prioritize the development of large-scale, optimally whole-wing, phenomic techniques that are compatible with quantization and focus on understanding the influence of signaling factors on time points relevant to SOP development and early stages of scale growth (McMillan *et al*. 2020).

A freedom that comes with viewing morphologies as processual outputs instead of discrete units is that it necessitates comparison and proposing linkages between structures. In a classic example, stepping back to view molluscan shell diversity not as wholly disparate evolutionary offshoots, but modifications of secretion along axes of whorl expansion, rate of translation, and relative position of the generating curve, permits constructing a parameter space in which all major shell types can be placed. More than this, all major shell types can be understood in relation to each other, and as differential outputs of the same three secretion vectors (Raup 1966). The so-called "Raup Cube" demonstrates one of the first examples of such 'morphospaces' and exemplifies their dual utility for both explanation and, more importantly, hypothesis generation. In the genetic era, morphospaces can be constructed with relative levels of signaling inputs as the sides to encapsulate variation in a structure across an evolutionary radiation, as has been demonstrated with beak traits among the Galápagos finches (Abzhanov *et al*. 2006). Since arthropod macrochaetae have already been demonstrated to occupy a continuous variation from one variety to the next, and variation in shape has been demonstrated to be influenced by genetic factors, I propose that instead of pretending to describe morphological variation of these structures as outputs of a deterministic genetic cascade, effort is put forward to understand and contextualize structural differences as exit ramps from a continuous process that itself is influenced by genetic or physical factors. While the latter may exist at the level of sensory organ types, it is, perhaps, more parsimonious an explanation to consider that such organization secedes to the latter later in morphogenesis. The difference here being that each cell in the former model is completely and strictly determined to become exactly its morphology by carefully balanced intrinsic inputs, whereas the latter explains intermediate morphologies as resulting from a receptivity to extrinsic, local agents.

Once relevant factors describing macrochaete variation are established, hypotheses of evolutionary trajectory and divergences among clades will become possible. In particular, it may allow mapping of homoplasy, and particularly, convergence of macrochaete phenotypes through the identification of shared inputs that shift morphologies to common scaffolds. A demonstration of this point using morphological data only across a clade of drosophilids was able to capture the increased diversification rates of genitalic characteristics and a joint comparison of morphology and standing variation between drosophilids and sepsid flies was able to suggest avenues for evolvability and larger scale macroevolutionary trends across wing venation traits as a property of proclaimed developmental bias (Sayad & Yassin 2019; Rohner & Berger 2023). It also will become possible to assess the principles and motifs by which variation is achieved within a structure. Already from purely morphological observations, it becomes tempting to implicate a role for heterochrony as a prevailing force in generating differences in macrochaete intermediates. Across species, setae, bristles, and scales seem to exist along a continuum where not only they grade into each other, as has already been pointed out, but this gradation correlates with overall size, exemplified in the setae series of the rice weevil, *Sitophilus oryzae*, or extent of elaboration on their surface, as beautifully showcased in plumed bristles of the collembolan *Proisotoma minuta* (Con.2B-D) (Massoud & Ellis 1977). In combination with the association of ploidy level and macrochaete size, this suggests that a factor influencing degree of endoreplication may be responsible for observed morphological gradients.

Whereas modern efforts are aiming to look at the expression of individual cells throughout a developmental series in an attempt to identify morphogenetic factors, if cell cycle regulation were to be the causal factor such methods as they currently exist would very likely not be sensitive enough to detect differences, lack the spatial information to recognize the presence of a gradient, or, at best, misattribute downstream factors as being causal. Therefore, parallel empirical efforts aiming to manipulate candidate processes would potentially prove more informative. Ironically, a simple experiment disrupting transcription through serial dosing with 5-fluorouracil in *Ephestia kuhniella* may prove to be the most illuminating in understanding scale morphogenesis as it identified four distinct stages of transcription that correlate to particular truncated scale phenotypes which align to the morphological stages in scale development captured in chapter I, namely 1) initial projection, 2) first extension, 3) cylindrical elongation, and 4) flattening (Muth 1965). The range of scale types, this would suggest, could result from merely pausing or halting development at any one of these “checkpoints”; furthermore, “pausing and playing” them along a scale’s growth, one could imagine, could create the more bizarre marginal scale types that proximally appear to be bristles before flattening distally. While heterochrony as a catch-all explanation for evolutionary developmental variation has been pooh-poohed, in this case the immense number of intermediate types along with the knowledge that scale types begin elongating at different points in development, nudges to some influence it may have on macrochaete form (Hanken 2015).

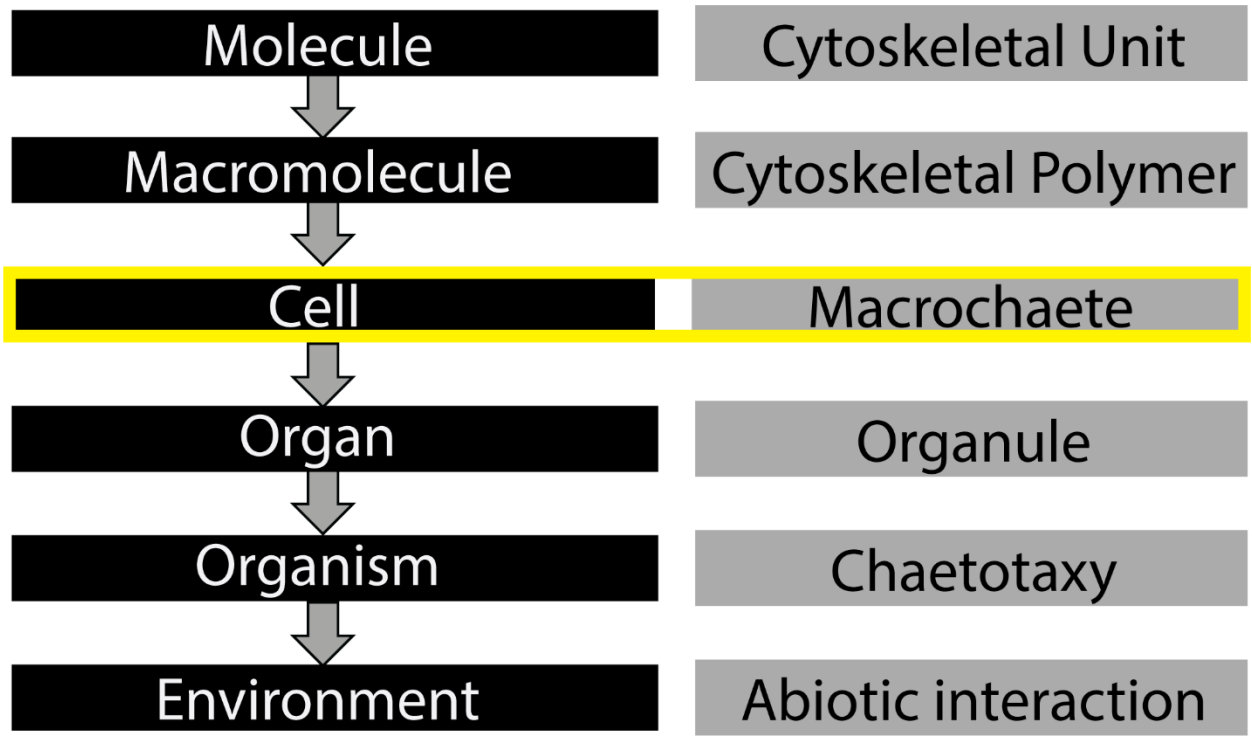
Concluding perspectives

It has been the aspiration in this dissertation research to tackle ideas in a way that draws the field forward and to create not only techniques but approaches others can follow. Insect macrochaetae as a system offer a wonderful playground with which to examine the generation and maintenance of biological diversity. Using the protocols outlined in this dissertation, it is possible to immediately start delving into the cell biology of arthropod exoskeletal projections, as exemplified in the case of taking mosquito pupae from local ponds and at once observe the dynamics of their scales' actin cytoskeleton (Con. 3).

The question of how convergent phenotypes arise is central to our understanding of the evolutionary process and gets close to the central question of the repeatability of the tape of life. Instead of approaches that solely aim to identify genetic regulators or switches between cell states, I believe it is richer and more rewarding to pursue the downstream elements that are acting in order to physically create the cells in question. In delving into the cytoskeletal systems of these cells, I have come to more deeply appreciate the linkages between levels of biological organization and now view diversity as a paradox of sameness stretched by contingency into perceived differences. Identifying factors which tweak cells into different forms continues to grow as an area of biological inquiry, and it is my hope that this work pushes arthropod macrochaetae further along in the fray.

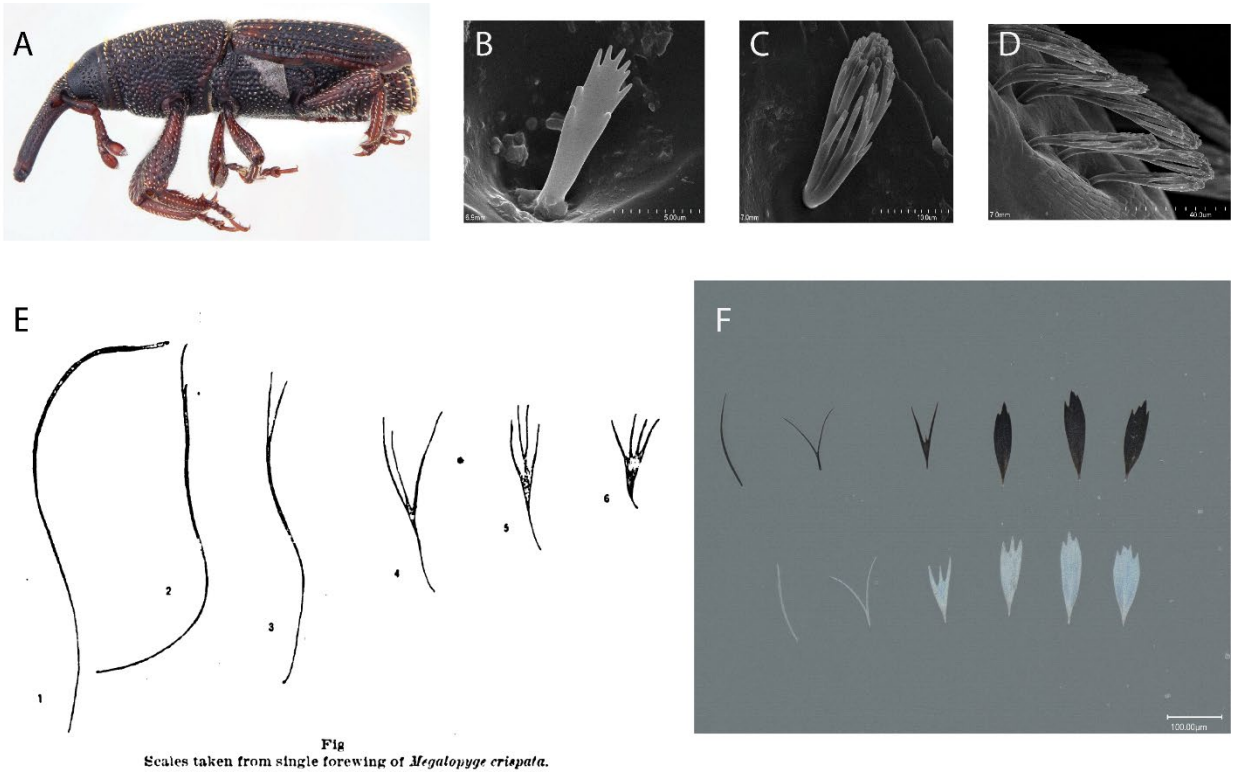
> *The great diversity of shape and size among the scales of moths and butterflies is a matter of common remark among entomologists. So nearly infinite, seemingly, are these variations that it is with hesitancy that one undertakes to find order in this chaos. But immensely greater diversity of structure has been found to resolve itself into approximate simplicity, and the "dust of the butterfly" is, after all, a more or less reasonable dust, and exhibits a tolerably rational behavior in its developmental caprices.* (p.46)
>
> -Vernon L. Kellogg
>
> From Kellogg 1894, *Kansas University Quarterly Vol. III*

Con. 1 Macrochaetae occupy a unique position to investigate biological organization



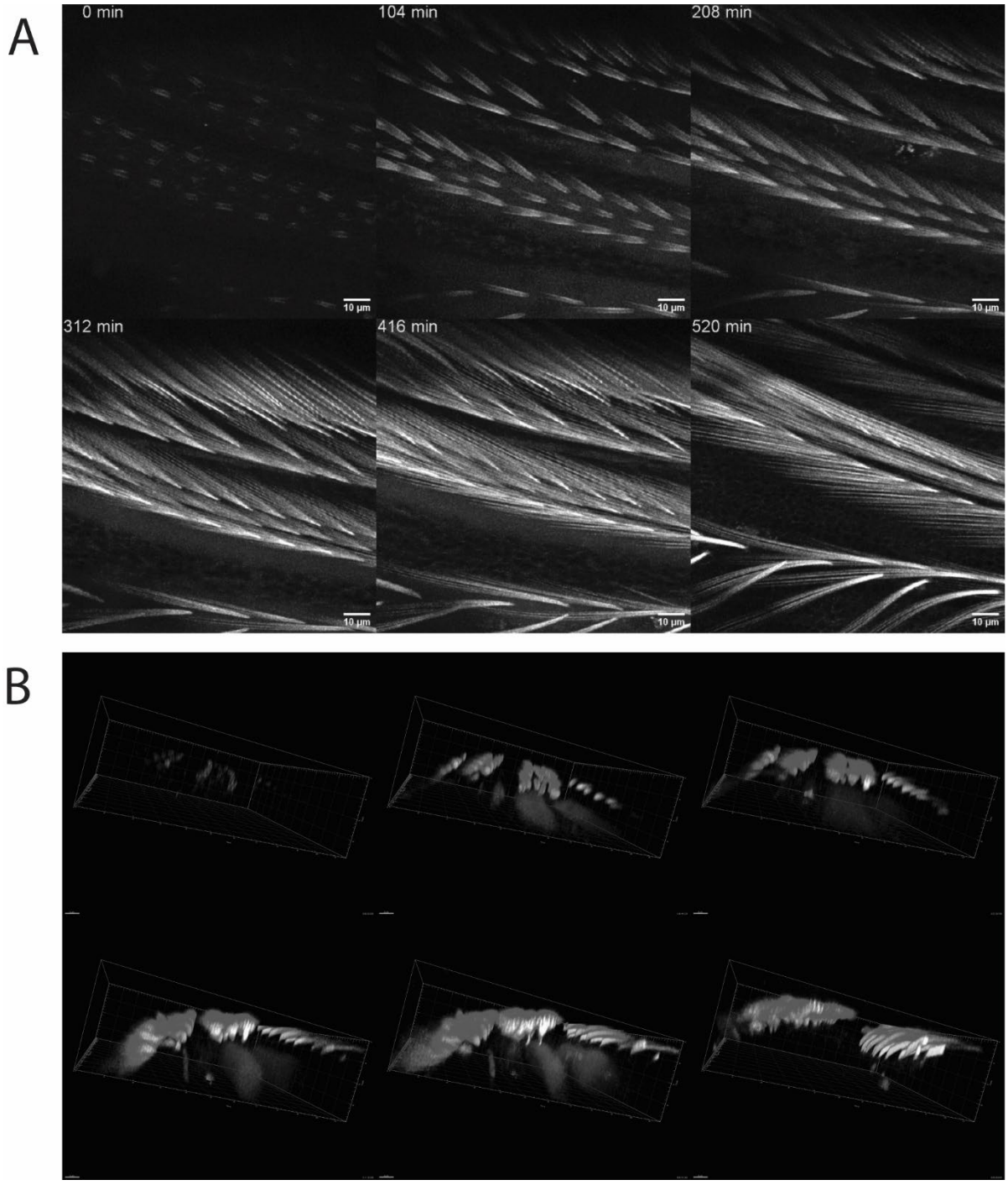
One example of a scheme matching elements of the hierarchy of biological organization (black boxes) with respective tiers (grey boxes) of the single cell-secreted macrochaete system (highlighted in yellow).

Con. 2 Scale morphologies as continua



Sitophilus oryzae female habitus focus stack (A) and macrochaete SEM micrographs representing lateral (B) and dorsal pronotal (C) and dorso-anterior circumocular (D) macrochaetae. Total scale bar lengths in B-D are 5µm, 10µm, and 40µm, respectively. SEM images taken on a Hitachi S-4700 at the American Museum of Natural History. *Sitophilus oryzae* provided by S.R. Davis. **E-** Fig. 10 from (Kellogg 1894) depicting "Scales taken from single forewing of *Megalopyge crispata*." Figure is in the public domain and obtained from the Biodiversity Heritage Library, contributed by *Smithsonian Libraries* (www.biodiversitylibrary.org). **F**- Series of black and white *Greta oto* scales taken from a linear gradient starting from a "clear" window region toward the scaled wing edge. Image taken with a Keyence VHX7000 at 300X magnification. Scale bar measures 100µm.

Con. 3 Actin organization in the growth of convergent mosquito scales



Culicidae sp. scale growth along wing veins in max projection (A) and xz "extending-toward-viewer" termed "head-on" (B) projections from approximately 6hrs after pupation ('00:00') to ~15hrs after pupation with scales labeled for filamentous actin with SiR-Act. Montage frames were sampled at 104min intervals from a movie where images were acquired every 8min. Scale bars in (A) measure 10µm and large tick marks on the bounding frame in (B) correspond to 1µm. Scale distal ends are pointed to the left of the frame (A) and toward the viewer (B).

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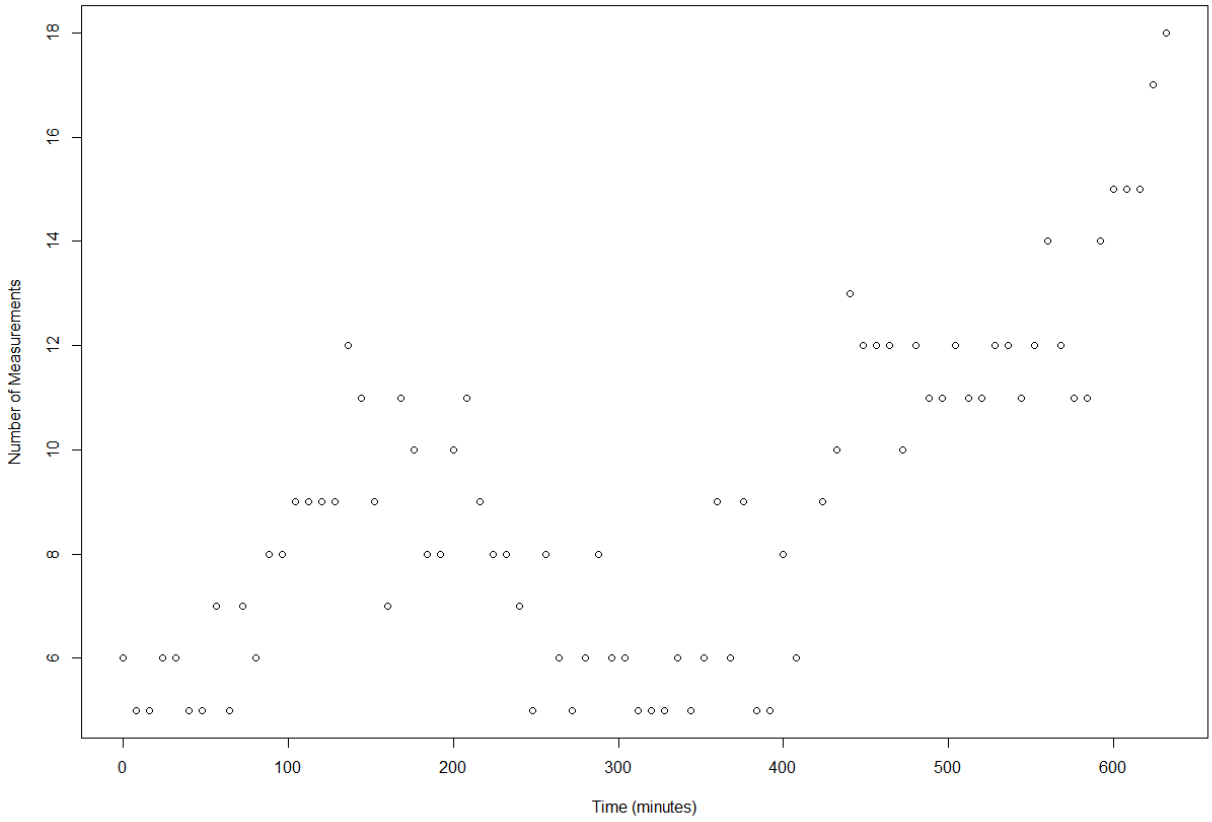
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Appendix I

Appendix I.1 Sample sizes of actin rod measurements

App. I.1 Plot of sample sizes for actin rod measurements from Fig.I.5.B



Plot of the number of actin rod width measurements vs time (minutes). Actin rod width values used to calculate means and standard deviations for each timepoint as displayed in Fig.I.5B.

Appendix I.2 Distal scale FRAP lines of best fit formulae

Formulae and R^2 values for the lines of best fit calculated for the distal FRAP and plotted in Fig.I.7.B. Output from Fiji.

```
Fit for Plot of Results: Data Set 1: 1 Positive Control

Formula: y = a+bx

Macro code: y = a+x*b

Status: Success

Number of iterations: 1

Time: 0 ms

Sum of residuals squared: 1.82345E9

Standard deviation: 3881.99084

R^2: 0.11575

Parameters:

      a = 25173.1786

      b = 39.71842

Fit for Plot of Results: Data Set 2: 2 Proximal

Formula: y = a+bx

Macro code: y = a+x*b

Status: Success

Number of iterations: 1

Time: 0 ms

Sum of residuals squared: 837747576

Standard deviation: 2631.26085

R^2: 0.66081

Parameters:

      a = 13660.9099

      b = 103.85742

Fit for Plot of Results: Data Set 3: 3

Formula: y = a+bx

Macro code: y = a+x*b

Status: Success

Number of iterations: 1
```

Time: 0 ms

Sum of residuals squared: 670330912

Standard deviation: 2353.70450

R^2: 0.80610

Parameters:

a = 8886.32432

b = 135.71302

Fit for Plot of Results: Data Set 4: 4

Formula: y = a+bx

Macro code: y = a+x*b

Status: Success

Number of iterations: 1

Time: 0 ms

Sum of residuals squared: 421753754

Standard deviation: 1866.96765

R^2: 0.76890

Parameters:

a = 6682.92330

b = 96.30172

Fit for Plot of Results: Data Set 5: 5

Formula: y = a+bx

Macro code: y = a+x*b

Status: Success

Number of iterations: 1

Time: 0 ms

Sum of residuals squared: 243845344

Standard deviation: 1419.59529

R^2: 0.60238

Parameters:

a = 4554.03452

b = 49.41102

Fit for Plot of Results: Data Set 6: 6 Negative Control

Formula: y = a+bx

Macro code: y = a+x*b

Status: Success

Number of iterations: 1

Time: 0 ms

Sum of residuals squared: 98849099.5

Standard deviation: 903.84440

R^2: 0.55798

Parameters:

a = 4268.46905

b = 28.71736

Appendix I.3 Central scale FRAP lines of best fit formulae

Formulae and R^2 values for the lines of best fit calculated for the central FRAP and plotted in Fig.I.7.D. Output from Fiji.

```
Fit for Plot of Results: Data Set 1: Positive Control
Formula: y = a+bx
Macro code: y = a+x*b
Status: Success
Number of iterations: 1
Time: 0 ms
Sum of residuals squared: 7.27337E9
Standard deviation: 3484.61252
R^2: 0.60630
Parameters:
      a = 44614.0103
      b = -24.94574
Fit for Plot of Results: Data Set 2: Proximal
Formula: y = a+bx
Macro code: y = a+x*b
Status: Success
Number of iterations: 1
Time: 1 ms
Sum of residuals squared: 428184409
Standard deviation: 845.47742
R^2: 0.94615
Parameters:
      a = 359.45931
      b = 20.44386
Fit for Plot of Results: Data Set 3: Distal
Formula: y = a+bx
Macro code: y = a+x*b
Status: Success
```

Number of iterations: 1

Time: 0 ms

Sum of residuals squared: 403592557

Standard deviation: 820.83934

R^2: 0.92078

Parameters:

a = 2968.94276

b = 16.14377

Fit for Plot of Results: Data Set 4: Negative Control

Formula: y = a+bx

Macro code: y = a+x*b

Status: Success

Number of iterations: 1

Time: 0 ms

Sum of residuals squared: 91153624.5

Standard deviation: 390.09785

R^2: 0.16592

Parameters:

a = 1146.68628

b = 1.00368

Appendix I.4 Scale aspect ratio imposed curves of best fit formulae: combined

Formulae and R^2 values for the imposed curves of best fit calculated for pooled scale aspect ratio measurements in each class and plotted in Fig.I.8.B. Output from Fiji.

```
Fit for Plot of Results: Data Set 1: Cover
Formula: y = a*exp(-bx) + c
Macro code: y = a*Math.exp(-b*x)+c
Status: Success
Number of completed minimizations: 4
Number of iterations: 208 (max: 1500)
Time: 16 ms
Sum of residuals squared: 12.35803
Standard deviation: 0.18605
R^2: 0.83976
Parameters:
      a = 0.26371
      b = -0.0043245
      c = 0.75868
Fit for Plot of Results: Data Set 2: Middle
Formula: y = a*exp(-bx) + c
Macro code: y = a*Math.exp(-b*x)+c
Status: Success
Number of completed minimizations: 4
Number of iterations: 190 (max: 1500)
Time: 31 ms
Sum of residuals squared: 30.26088
Standard deviation: 0.30656
R^2: 0.70486
Parameters:
      a = 0.0097223
      b = -0.011465
      c = 1.29533
```

Fit for Plot of Results: Data Set 3: Ground

Formula: y = a*exp(-bx) + c

Macro code: y = a*Math.exp(-b*x)+c

Status: Success

Number of completed minimizations: 4

Number of iterations: 210 (max: 1500)

Time: 31 ms

Sum of residuals squared: 9.46871

Standard deviation: 0.19500

R^2: 0.55180

Parameters:

a = 9.52032E-6

b = -0.025159

c = 1.41050

Appendix I.5 Scale aspect ratio imposed curves of best fit formulae: individual

Formulae and R^2 values for the imposed curves of best fit calculated for individual scale aspect ratio measurements in each class and plotted in Sup.I.6.A-E. Output from Fiji.

```
Fit for Plot of Results: Data Set 1: CoverAR1
Formula: y = a*exp(-bx) + c
Macro code: y = a*Math.exp(-b*x)+c
Status: Success
Number of completed minimizations: 4
Number of iterations: 210 (max: 1500)
Time: 10 ms
Sum of residuals squared: 3.76495
Standard deviation: 0.17713
R^2: 0.85795
Parameters:
      a = 0.62541
      b = -0.0028808
      c = 0.22056
Fit for Plot of Results: Data Set 2: CoverAR2
Formula: y = a*exp(-bx) + c
Macro code: y = a*Math.exp(-b*x)+c
Status: Success
Number of completed minimizations: 4
Number of iterations: 228 (max: 1500)
Time: 10 ms
Sum of residuals squared: 2.29975
Standard deviation: 0.13844
R^2: 0.88385
Parameters:
      a = 0.11068
      b = -0.0057755
      c = 1.03126
```

Fit for Plot of Results: Data Set 3: CoverAR3

Formula: y = a*exp(-bx) + c

Macro code: y = a*Math.exp(-b*x)+c

Status: Success

Number of completed minimizations: 4

Number of iterations: 207 (max: 1500)

Time: 9 ms

Sum of residuals squared: 4.06926

Standard deviation: 0.18811

R^2: 0.85834

Parameters:

a = 0.22444

b = -0.0047946

c = 0.85988

Fit for Plot of Results: Data Set 4: MiddleAR1

Formula: y = a*exp(-bx) + c

Macro code: y = a*Math.exp(-b*x)+c

Status: Success

Number of completed minimizations: 4

Number of iterations: 220 (max: 1500)

Time: 20 ms

Sum of residuals squared: 1.61226

Standard deviation: 0.11591

R^2: 0.91813

Parameters:

a = 0.0088654

b = -0.011249

c = 1.11654

Fit for Plot of Results: Data Set 5: MiddleAR2

Formula: y = a*exp(-bx) + c

Macro code: y = a*Math.exp(-b*x)+c

Status: Success

Number of completed minimizations: 4

Number of iterations: 224 (max: 1500)

Time: 11 ms

Sum of residuals squared: 3.00505

Standard deviation: 0.17976

R^2: 0.92262

Parameters:

a = 0.0060118

b = -0.013102

c = 1.54128

Fit for Plot of Results: Data Set 6: MiddleAR3

Formula: y = a*exp(-bx) + c

Macro code: y = a*Math.exp(-b*x)+c

Status: Success

Number of completed minimizations: 4

Number of iterations: 212 (max: 1500)

Time: 5 ms

Sum of residuals squared: 3.47314

Standard deviation: 0.18187

R^2: 0.80573

Parameters:

a = 0.0016822

b = -0.014823

c = 1.46465

Fit for Plot of Results: Data Set 7: GroundAR1

Formula: y = a*exp(-bx) + c

Macro code: y = a*Math.exp(-b*x)+c

Status: Success

Number of completed minimizations: 4

Number of iterations: 206 (max: 1500)

Time: 15 ms

Sum of residuals squared: 1.16047

Standard deviation: 0.11896

R^2: 0.86472

Parameters:

a = 2.83926E-5

b = -0.023385

c = 1.25122

Fit for Plot of Results: Data Set 8: GroundAR2

Formula: y = a*exp(-bx) + c

Macro code: y = a*Math.exp(-b*x)+c

Status: Success

Number of completed minimizations: 4

Number of iterations: 210 (max: 1500)

Time: 8 ms

Sum of residuals squared: 3.08788

Standard deviation: 0.19173

R^2: 0.45917

Parameters:

a = 0.00029236

b = -0.016999

c = 1.53171

Fit for Plot of Results: Data Set 9: GroundAR3

Formula: y = a*exp(-bx) + c

Macro code: y = a*Math.exp(-b*x)+c

Status: Success

Number of completed minimizations: 4

Number of iterations: 202 (max: 1500)

Time: 8 ms

Sum of residuals squared: 1.94224

Standard deviation: 0.15485

R^2: 0.56622

Parameters:

a = 2.03346E-7

```
b = -0.033467
c = 1.41204
```

Appendix II

Appendix II.1 ACME protocol for dissociation of lepidopteran pupal wings

Solutions

Deionized H_2O

Methanol (A412-1 Fisher Scientific: https://www.fishersci.com/shop/products/methanol-certified-acs-fisher-chemical-10/A4121#?keyword=)

Glacial Acetic Acid (A6283-1L Sigma-Aldrich: https://www.sigmaaldrich.com/US/en/product/sigald/a6283?context=product)

Glycerol (G33-500 Fisher Scientific: https://www.fishersci.com/shop/products/glycerol-certified-acs-fisher-chemical-6/G33500#?keyword=)

Bovine Serum Albumin Fraction V (#10735078001 Sigma-Aldrich: https://www.sigmaaldrich.com/US/en/product/roche/bsavro)

DAPI (1mg/mL in H_2O)

Propidium Iodide (1mg/mL in H_2O)

Phosphate Buffered Saline (PBS; pH=7.4)

Materials

15mL Falcon high-clarity polypropylene tubes (Corning, #352096)

7mL plastic transfer pipets, graduated to 3mL (Globe Scientific Inc., #135030)

P1000 + tips (Genesee, Reach Barrier Tips, low-binding, pre-sterilized, Cat# 24-430)

P200 + tips (Genesee, Pre-Sterilized, Cat#24-151RS)

Glass microscope slides

#1.5 glass coverslips

Equipment

Platform shaker (capable of at least 70rpm; protocol tested with Innova 2000)

Temperature-controlled Centrifuge (buckets compatible with 15mL conical; capable of 2,000G at 4°C; protocol tested with Sorvall Legend RT)

Compound microscope with fluorescence (DAPI and TexasRed) and 10X, 20X, 40X objectives

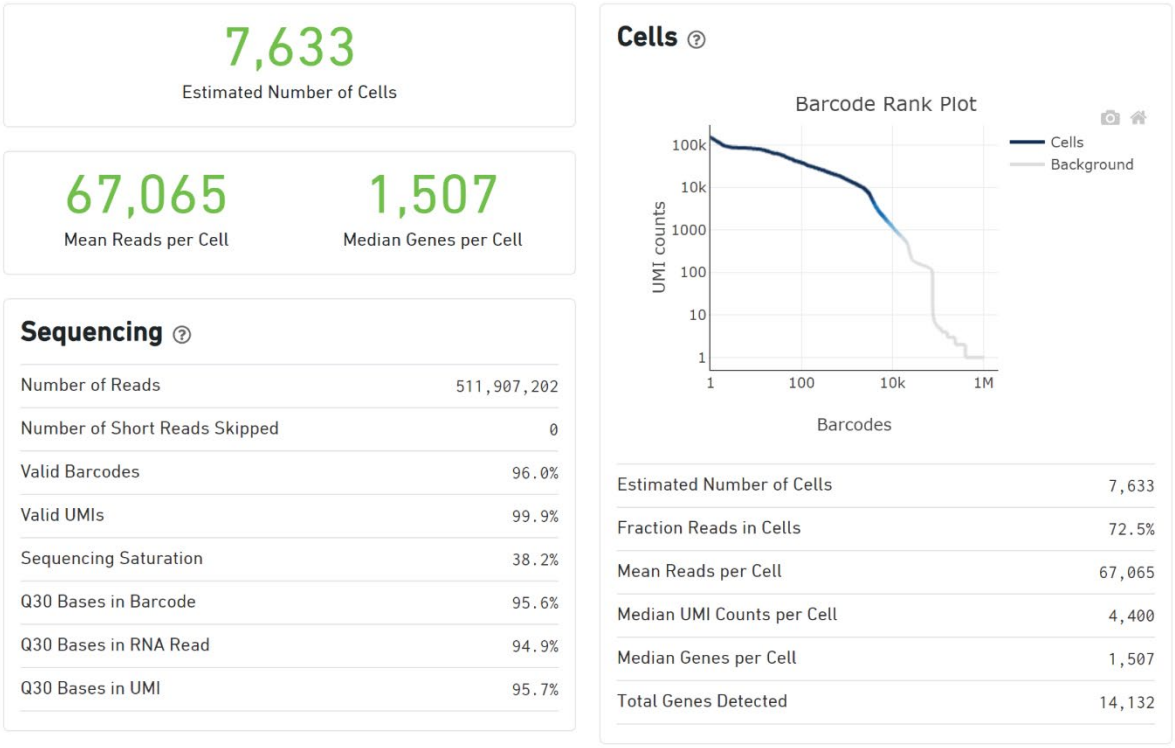
(Optional) 41µm Nylon filter (EMD Millipore Corp., NY4102500)

Method

1. Prepare fresh ACME (ACetic-MEthanol) dissociation solution before every set of dissections. Using 7mL plastic transfer pipets, combine 13 parts deionized water, 3 parts methanol, 2 parts glacial acetic acid, and 2 parts glycerol to a volume of 10mL (6.5mLH_2O:1.5mLMeOH:1mLHOAc:1mLGly) into a 15mL conical and invert several times to mix thoroughly.
2. Dissect *Junonia coenia* hindwings quickly in chilled PBS and place in freshly prepared ACME solution as you remove them from the membrane. Wings kept in PBS for too long will shrivel which could compromise cell health and expression profile.
3. Invert conical several times to completely immerse hindwings. Wait five minutes. Thoroughly pipet ACME solution and wing with a P1000. Continue pipetting until most large chunks of wing have vanished.
4. Place conicals of cell suspension in ACME on a platform shaker at 70rpm for one hour. Remove at fifteen-minute intervals and repeat thorough pipetting. After the second round, no large chunks should be visible.
5. After four rounds of mechanical dissociation with the P1000 and an hour total on the platform shaker, remove conical with fully suspended cells and centrifuge at 2000G and 4°C for five minutes. A pink/tan pellet of cells should clearly form at the bottom of the conical.
6. Pipet off ACME fixation supernatant carefully, leaving the pellet intact. Add 7mL of chilled PBS + 1% Bovine Serum Albumin (.07mg per 7mL solution, shake thoroughly and leave or centrifuge for foam to settle). Flick to completely dissociate pellet.
7. Centrifuge cell suspension at 2000G and 4°C for five minutes. A pink/tan pellet of cells should again clearly be visible on the bottom of the conical.
8. Carefully pipet off PBS + 1%BSA supernatant and resuspend cells in 75µL chilled PBS.
9. (Optional) This step is recommended for older pupal wings with more mature scales that break at the pedicel from cell bodies, and thus higher amounts of suspension debris. Add 100µL of chilled PBS to resuspended cell mixture and pass resuspended cells through a 41µm Nylon filter into an Eppendorf tube. Centrifuge cell suspension at 2000G and 4°C for five minutes and resuspend resultant pellet by flicking the tube, as before.
10. Add .5µL 1mg/mL DAPI and .5µL 1mg/mL propidium iodide to cell suspension, flicking to distribute dye. Wait ten minutes for cells to stain.
11. Pipet up and down and remove 50µL of stained cell suspension. Mount on a microscope slide with #1.5 coverslips. Image under fluorescence on a compound microscope with 10X, 20X, and 40X magnification to determine cell size and concentration of the cellular suspension.

Appendix II.2 Cell Ranger QC Output from scRNAseq

App. II.1 Cell Ranger QC output from scRNAseq of *Junonia coenia*



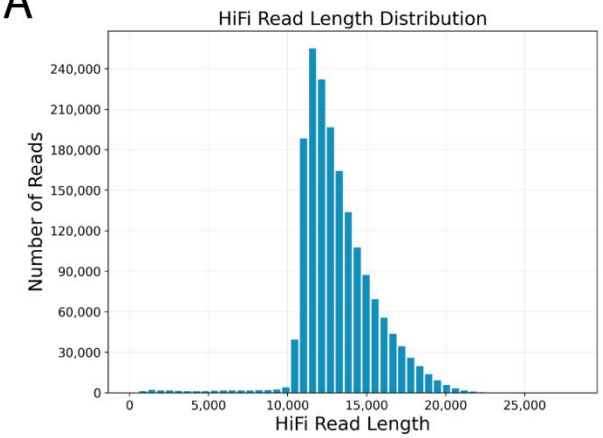
Output from 10X CellRanger web interface for quality check of the *Junonia coenia* scRNAseq run based on the *J. coenia* 'JC v 1.0' genome (van der Burg *et al*. 2019).

Appendix III

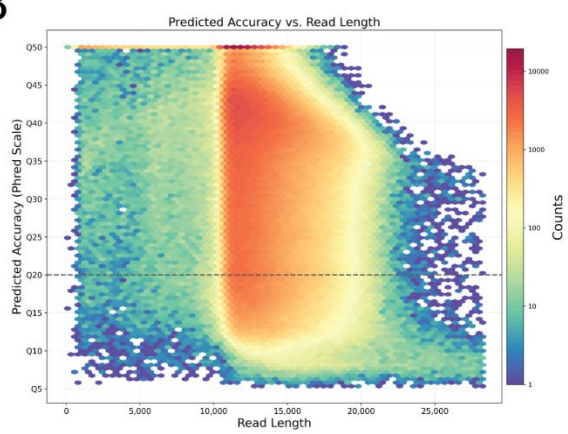
Appendix III.I Statistics for *Nectopsyche exquisita* genome

App. III.1 Genome statistics for *Nectopsyche exquisita* from PacBio HiFi sequencing

A



B



C

Value	
1,718,585	HiFi Reads
22,638,593,827	HiFi Yield (bp)
13,172	HiFi Read Length (mean, bp)
Q36	HiFi Read Quality (median)
14	HiFi Number of Passes (mean)
294,489	<Q20 Reads
4,133,854,744	<Q20 Yield (bp)
14,037	<Q20 Read Length (mean, bp)
Q16	<Q20 Read Quality (median)

A- Number of reads vs HiFi Read length plot, **B-** Predicted accuracy of reads vs read length plot, **C-** Collected sequence values for HiFi run including total read number, total basepairs generated, mean read length, median read quality, mean HiFi passes, and <Q20 reads, yield, mean read length, and median read quality.