ANNUAL
REVIEWS

Annual Review of Entomology

The Developmental Origin of Novel Complex Morphological Traits in Lepidoptera

Antónia Monteiro,[1,*] Suriya Narayanan Murugesan,[1]
Anupama Prakash,[2] and Riccardo Papa[3,4,5]

[1]Department of Biological Sciences, National University of Singapore, Singapore;
email: antonia.monteiro@nus.edu.sg

[2]School of Biosciences, University of Sheffield, Sheffield, United Kingdom

[3]Department of Biology and Molecular Sciences Research Center, University of Puerto Rico, San Juan, Puerto Rico

[4]Dipartimento di Scienze Chimiche della Vita e della Sostenibilità Ambientale, Università di Parma, Parma, Italy

[5]Comprehensive Cancer Center, University of Puerto Rico, San Juan, Puerto Rico

Annu. Rev. Entomol. 2025. 70:421–39

The *Annual Review of Entomology* is online at ento.annualreviews.org

https://doi.org/10.1146/annurev-ento-021324-020504

*Corresponding author

Keywords

gene-regulatory networks, novelties, wing patterns, abdominal legs, scales, co-option

Abstract

Novel traits in the order Lepidoptera include prolegs in the abdomen of larvae, scales, and eyespot and band color patterns in the wings of adults. We review recent work that investigates the developmental origin and diversification of these four traits from a gene-regulatory network (GRN) perspective. While prolegs and eyespots appear to derive from distinct ancestral GRNs co-opted to novel body regions, scales derive from in situ modifications of a sensory bristle GRN. The origin of the basal and central symmetry systems of bands on the wing is associated with the expression of the *WntA* gene in those regions, whereas the more marginal bands depend on two other genes, *Distal-less* and *spalt*. Finally, several genes have been discovered that play important roles in regulating background wing color, via the regulation of pigmentation GRNs. The identification of shared and novel *cis*-regulatory elements of genes belonging to these distinct GRNs helps trace the developmental and evolutionary history of these traits. Future work should examine the extent to which ancestral GRNs are co-opted/modified to produce the novel traits and how these GRNs map to specific cell types in ancestral and derived traits.

INTRODUCTION

Lepidopterans, commonly known as butterflies and moths, have a variety of novel morphological traits that emerged within the clade or in its immediate ancestors. These insects have chubby abdominal prolegs that help the larvae move and grab onto substrates; scales on their wings, which give the order its name; and a variety of distinct and complex wing color patterns, including eyespots and bands, made from the spatial arrangement of scales of different colors. How these and other morphological novelties originate in lepidopterans, and in any other organism, is an active area of research.

In general, most morphological novelties are likely produced by the activation of preexisting regulatory genes at novel cellular locations in the body (18, 89). Because developmental genes are often embedded within complex gene-regulatory networks (GRNs), when individual genes within these networks are expressed in novel body locations, or cell types, they might be able to activate a similar set of genomic targets at these locations, as they did at the ancestral site (58) (**Figure 1**). Depending on the gene's position within the GRN, a novel expression for an individual gene in a new cell type may thus produce either duplicate traits, also termed serial homologs, or novel traits (55). The production of a serial homolog may indicate that the gene recruitment happened at a very high level in the GRN, perhaps at the level of a master regulator, whereas traits that differ considerably from preexisting traits could indicate that a partial GRN recruitment took place instead (60, 61) (**Figure 1**). In addition, the evolution of novel regulatory interactions of the GRN with spatially restricted transcription factors (TFs) can help diversify an ancestral GRN into a distinct GRN in the domain of those TFs (**Figure 1**).

In the sections that follow, we review recent studies that illustrate both the creation and the modification of serial homologs at the molecular or developmental level in the bodies of lepidopterans. These animals are emerging as exceptional systems for molecular investigations of this

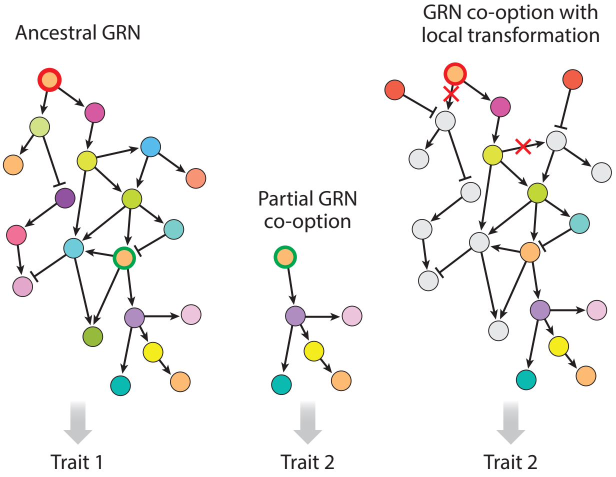


Figure 1

Conceptual framework for the evolution of novel traits via gene-regulatory network (GRN) co-option. A novel trait (Trait 2) can evolve from the partial expression of a preexisting GRN (coding for Trait 1) in a novel developmental context in the same body—here represented by the novel expression of the gene outlined in green in a novel context. Alternatively, a novel trait can derive from the expression of a top GRN regulator (gene outlined in *red*) in a new context. Novelty (rather than serial repetition) might arise if context-specific genes (*red circles*) alter the expression and output of the network in the novel context (*gray circles* represent inactive genes).

GENOME RESOURCES AND FUNCTIONAL GENETIC TOOLS FOR LEPIDOPTERANS

Over 300 lepidopteran genomes have been sequenced. They can be found at the following locations:

- Lepbase (12): **http://ensembl.lepbase.org/index.html**
- Darwin Tree of Life (16, 103): **https://portal.darwintreeoflife.org/**
- NCBI genome data sets: **https://www.ncbi.nlm.nih.gov/datasets/genome/?taxon=7088**

A few CRISPR-Cas9 genome editing protocols for lepidopterans have been published:

- Zhang & Reed (107)
- Banerjee & Monteiro (2)
- Li et al. (41)

Examples of lepidopterans for which CRISPR-Cas9 has been employed:

- *Agraulis vanillae*
- *Bicyclus anynana*
- *Bombyx mori*
- *Colias eurytheme*
- *Danaus plexippus*
- *Heliconius* spp. (*cydno*, *erato*, *hewitsoni*, *himera*, *pachinus*, *sapho*, *sara*)
- *Helicoverpa armigera*
- *Junonia coenia*
- *Plodia interpunctella*
- *Papilio xuthus*
- *Pieris canidia*
- *Plutella xylostella*
- *Vanessa cardui*

nature given the number of genomes sequenced and the development of functional genetic tools across multiple species (see the sidebar titled Genome Resources and Functional Genetic Tools for Lepidopterans). Future research that cements and probes these models further is required and is highlighted at the end of the review.

ORIGIN OF PROLEGS IN THE LEPIDOPTERA: RECRUITMENT OF AN EPIPOD GRN

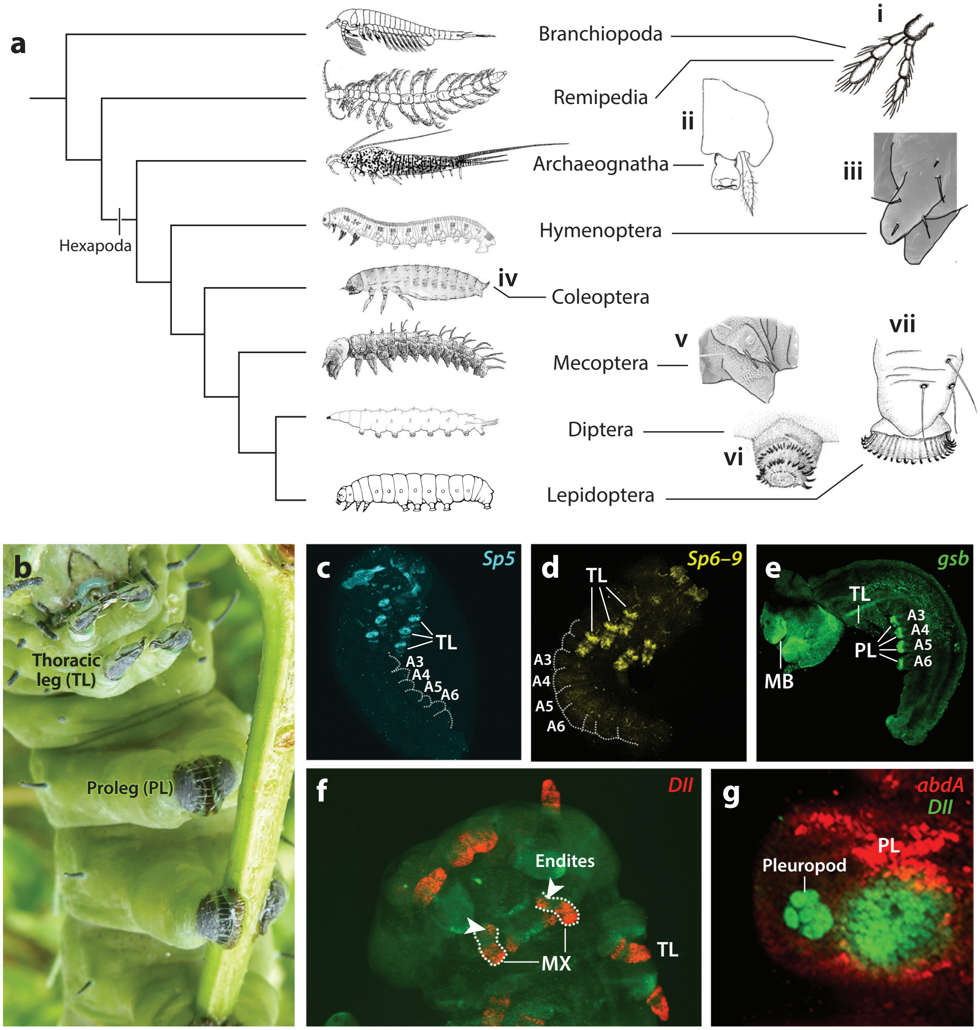
Most adult Hexapoda, to which Lepidoptera belong, have three pairs of thoracic legs and no other walking limbs in the abdomen. The leg GRN, expressed in the abdomen of close relatives such as remipeds and branchiopods (28) (**Figure 2*a***), has been repressed in the abdomen of hexapods by *abdominal A* (*abdA*), a Hox gene expressed in abdominal segments (39, 91). Fleshy abdominal appendages, however, are present in the adults of nonwinged insects such as Archaeognatha (83), as well as in several larval hexapods (**Figure 2*a***). These include the Lepidoptera and its sister lineage, the Diptera (22); sawfly larvae (Hymenoptera) (85); and scorpionfly embryos (Mecoptera) (37, 42, 104). Whether these appendages evolved once, in the common ancestor of all Hexapoda, or multiple times independently will determine whether they are homologous or convergent traits.

The appendages present in the abdomen of Lepidoptera are called prolegs. They are used for walking and grabbing onto branches while the larva feeds with the help of its thoracic legs

(**Figure 2*b***). Unlike thoracic legs, these prolegs lack a claw and typically display a row of small hooks at the tip, which help the larva grab onto surfaces.

Several hypotheses (10, 32, 82; reviewed in 51) regarding the developmental nature of these prolegs proposed that they were either thoracic leg serial homologs that contained only the most proximal segments, or the most distal elements, novel traits all together, or proximal lobes of legs (endites) once present in the ancestors of hexapods but currently retained only in mouthparts. A recent study tested these several hypotheses and found that prolegs are likely a combination of the last two (51).

Prolegs appear to be novel traits that have co-opted the endite GRN to the abdomen via a novel regulatory input. They do not appear to be truncated thoracic legs because they do not express thoracic leg marker genes, such as *Sp5* and *Sp6–7*, including in the most proximal segments (**Figure 2*c*,*d***). An endite marker gene, however, *paired* (a paralog of *gooseberry* in crustaceans), was expressed in prolegs and in endites of the maxilla and the mandible (**Figure 2*e***). *Distal-less*



(*Caption appears on following page*)

Figure 2 (*Figure appears on preceding page*)

The development and evolution of lepidopteran prolegs (PLs). (*a*) The presence of abdominal appendages in hexapods. Unlike its sister lineages, the Branchiopoda and Remipedia, which have biramous appendages at nearly every segment of the body (*i*), a variety of more subtle abdominal appendages are found across the Hexapoda, whose developmental nature is still unclear. Species depicted include (*ii*) an adult Archaeognatha with eversible vesicles and styli (cross-section); (*iii*) a sawfly larvae with PLs (Hymenoptera); (*iv*) the coleopteran *Tribolium castaneum*, which has small abdominal nubs only as an embryo; (*v*) a mecopteran embryo (*Panorpa magna*) just before hatching; (*vi*) an Athericidae dipteran larvae with pseudopods or PLs; and (*vii*) a lepidopteran larva with PLs. (*b*) Legs and PLs of the Atlas moth caterpillar (*Attacus atlas*). (*c–e*) PLs express genes that are distinct from those expressed by thoracic legs (TLs) during development of *Bicyclus anynana* butterflies. (*c*) Expression of *Sp5* and (*d*) *Sp 6–9* is seen only in TLs, not PLs. (*e*) Expression of *gooseberry* (*gsb*) is present in PLs and mandibles (MBs) but not in TLs. (*f*) *Distal-less* (*Dll*) is a leg, PL, and endite marker gene (*arrowheads*), here expressed in the distal tip and inner lobe (endite) of the maxillae (MX) of a *B. anynana* embryo. (*g*) Partial knockout of *abdominal A* (*abdA*) leads to the derepression of pleuropods that can develop next to PLs in the same segment of *B. anynana* embryos. Image credits for the illustrations on the left side of panel *a* are as follows: Branchiopoda illustration reproduced from Reference 78. Remipedia illustration reproduced with permission from Reference 20; copyright San Diego Natural History Museum. Archaeognatha illustration reproduced from Hallvard Elven/Natural History Museum, University of Oslo (CC BY 3.0). Hymenoptera illustration reproduced from Randall Blackburn, Smithsonian Institution, Bugwood.org (**https://www.bugwood.org/insects/sawflies.cfm**) (CC BY 4.0). Coleoptera illustration reproduced with permission from Reference 1. Mecoptera illustration adapted from Reference 104. Diptera illustration reproduced from Reference 22. Lepidoptera illustration from **https://commons.wikimedia.org/wiki/File:External_morphology_of_the_caterpillar_RUS.png** (public domain). Subpanel *ii* reproduced with permission from Reference 83. Subpanel *iii* adapted from Reference 109. Subpanel *iv* reproduced from Reference 87. Subpanel *v* reproduced from Reference 104. Subpanel *vi* reproduced with permission from Reference 84. Subpanel *vii* reproduced with permission from Reference 77. Panels *b–g* modified from Reference 51 (CC BY 4.0).

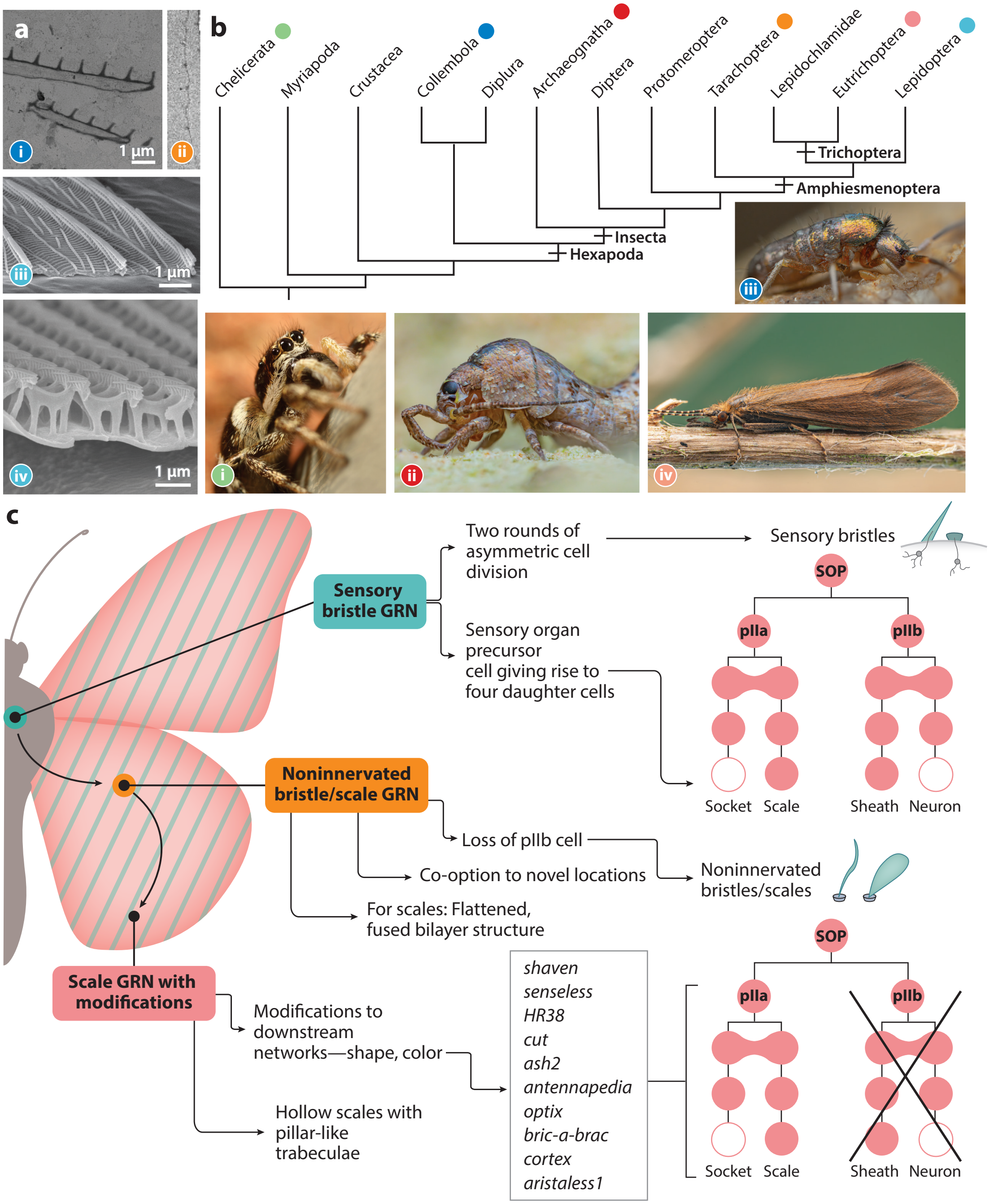
(*Dll*), whose expression in prolegs was initially used as evidence for prolegs being thoracic leg homologs (97), is also an endite marker gene (**Figure 2*f***); so it alone cannot distinguish the two GRNs. Furthermore, the expression of *Dll* in proleg and leg domains appears to be driven by distinct *cis*-regulatory elements (CREs) (51, 60). Most importantly, mosaic *abdA* knockouts, which removed the gene's function from more lateral abdominal regions of larvae, led to the development of two traits side by side: pleuropods, which are thoracic leg homologs normally repressed by *abdA*, and prolegs (in the more medial region), which require *abdA* input for their development (51) (**Figure 2*g***). This research showed that the same Hox gene that had been implicated in the repression of thoracic legs/pleuropods in the ancestors of Hexapoda was now involved in activating a distinct GRN, the endite GRN, in a more medial location to produce prolegs. Furthermore, comparison of the transcripts from prolegs to those from multiple other larval body appendages suggested that the co-option of the endite GRN might have happened more than once to create another novel trait in Lepidoptera—head horns (51).

Recent work in mecopteran embryos containing prolegs also found that *abdA* promotes proleg development in these holometabolous insects (42). Furthermore, these prolegs developed along the same medial line as maxillary endites present in the head appendages (42). This finding suggests that prolegs in Lepidoptera and Mecoptera may be homologous at the developmental level.

Future work should examine the detailed development of the abdominal appendages of other hexapods depicted in **Figure 2*a*** to test whether these appendages are using the same endite GRN as lepidopteran prolegs or distinct GRNs. This work will help determine whether these appendages are process homologous.

ORIGIN AND DEVELOPMENT OF SCALES IN LEPIDOPTERA: MODIFICATION OF A SENSORY BRISTLE GRN

The Lepidoptera are a unique lineage where wings are covered by a dense layer of scales. Scales are noninnervated structures with a short stalk that are attached to the underlying surface via a socket (19). Scales can be morphologically diverse, resembling flat paddles, forked structures, or thin hairlike bristles. These rigid structures are made from cuticle, consisting of chitin polymers, cuticular proteins, and pigments secreted to the outside of the cell during pupal development, which hardens during adult emergence (27, 43). Flat scales have two surfaces—an upper lamina and a lower lamina—which are either juxtaposed to create a fused, solid scale (26) (**Figure 3*a***,



(*Caption appears on following page*)

Figure 3 (*Figure appears on preceding page*)

The development and evolution of lepidopteran scales. (*a*) Electron micrographs of scales from (*i*) a springtail, *Tomocerus vulgaris* (Collembola) (subpanel reproduced with permission from Reference 95); (*ii*) an amber fossil, *Kinitocelis brevicostata* (Tarachoptera) [subpanel reproduced with permission from Reference 108 (CC BY-NC 1.0)]; (*iii*) a basal Lepidoptera, *Micropterix aureatella* (Micropterigidae) (subpanel reproduced with permission from Reference 35); and (*iv*) a derived Lepidoptera, *Junonia orithya* (Nymphalidae). (*b*) Scales are present on the bodies of many arthropods, such as (*i*) *Salticus scenicus* (Chelicerata), (*ii*) *Dilta* sp. (Archaeognatha), (*iii*) *Tomocerus* sp. (Collembola), and (*iv*) Eutrichoptera. Photo in subpanel *i* reproduced with permission; copyright J. Paul Richards. Photos in subpanels *ii–iv* reproduced with permission; copyright Tim Jonas. The colored circles in panels *a* and *b* correspond to the different lineages in the phylogeny. (*c*) A schematic of the sensory bristle and the noninnervated bristle/scale cell lineages, including a possible evolutionary trajectory and GRN modifications of these structures from the bodies of insects to coverage of the entire wing. The box contains a nonexhaustive list of genes that play important roles in scale specification and diversification. Abbreviations: GRN, gene-regulatory network; SOP, sensory organ precursor.

subpanels *i–iii*) or connected to each other via pillar-like trabeculae to create hollow scales (**Figure 3*a*, subpanel *iv***). The lower lamina is often a smooth thin film, whereas the upper lamina is structured with ridges and cross-ribs.

Scales, which were initially hypothesized to have evolved on the wings of lepidopterans, appear to have evolved earlier, in the common ancestor of the superorder Amphiesmenoptera, containing the Tarachoptera (an extinct lineage), Trichoptera, and Lepidoptera (96) (**Figure 3*b***). Amber fossils show that scales in the Tarachoptera, the most basal-branching lineage within Amphiesmenoptera, and in a basal lineage of Trichoptera (Lepidochlamidae) were of two types: either bristlelike (108) or flat, spindle-shaped, and fused, with a sharp apex, having ridges but no cross-ribs (96). Flat scales were then almost completely lost in the Trichoptera, where wings are largely covered by noninnervated bristlelike scales (**Figure 3*b***). Flat scales then became wider with rounded apical margins and acquired cross-ribs at the base of the Lepidoptera (96).

The developmental evolution of wing scales is still poorly understood but likely followed a multistep process that involved the co-option, modification, and diversification of the sensory bristle GRN. Galant et al. (23) hypothesized that during the evolution of the Amphiesmenoptera, genetic changes upstream and downstream of the *achaete-scute* complex (AS-C) genes that control early processes of sensory bristle development led to noninnervated bristles covering the entire wing. Further modifications in the targets of AS-C in lepidopterans led to changes in pigmentation and cytoskeletal features to produce flat scales. However, occurrences of both bristlelike and flat scales in Tarachoptera (108) suggest that the initial diversification of multiple noninnervated structures covering the wing happened at the base of the Amphiesmenoptera. Flat scales were lost in most Eutrichoptera, though some species like *Lepidostoma hirtum* retained flat scales on their wings.

The evolution of scales within the Lepidoptera was also a stepwise process, where scale shape diversified extensively and a bilayer system of ground and cover scales evolved (81, 96, 108). Basal lepidopterans, such as micropterigids, have fused scales with rounded apical margins (15, 35, 94, 108) (**Figure 3*a*, subpanel *iii***). More derived lepidopterans possess hollow scales with either rounded or dentate apical margins (94) (**Figure 3*a*, subpanel *iv***). These hollow scales originated via the development of pillars (trabeculae) inside the scale, which separated the upper and lower laminae, creating a space in between them, the lumen. The appearance of the scale lumen potentially increased the gamut of lepidopteran scale colors by creating space for the incorporation of pigment granules (30) and additional scale elaborations, such as multiple layers of cuticle or complex 3D cuticular depositions, producing photonic crystals (69, 100). The lumen also created an air layer implicated in the broadband reflectance of metallic scales (71, 74). These derived and modified hollow scales created some of the brightest structural colors in the natural world (86).

Both scales and sensory bristles share similar developmental progression and gene expression patterns (19, 23, 70), but the GRNs have diverged in a few key places. In sensory bristles, a single sensory organ precursor cell undergoes two rounds of asymmetric cell division to produce four daughter cells, whereas in scales, one of the daughter cells in the first round dies, leading to the loss of the sheath and neuron cells (76) (**Figure 3*c***). Genes such as *shaven*, *cut*, and *senseless* are expressed in both cell lineages (11, 25, 34, 64, 70) (**Figure 2*c***; see the sidebar titled Genome Resources and Functional Genetic Tools for Lepidopterans), but divergence in downstream targets likely led to scale cell type diversification (70). A hormone receptor, *HR38*, is necessary for the development of bristlelike scales because knockouts of the gene eliminated this specific scale cell type (70). Further, while bundles of actin filaments specify the locations of ridge features in scales (17, 19), recent work has shown extensive rearrangements of actin filaments at later stages of scale development that pattern dentate scale edges, cross-ribs, and honeycomb lattices between ridges (46, 79). The presence of genes that distinguish bristles from scales, and scales from each other, indicates that the sensory bristle GRN evolved and diversified via the recruitment of these modifiers to subsets of the serially repeated units.

Sensory bristle diversification into scales may have happened multiple times independently, in organisms outside the Amphiesmenoptera (29, 105), and scales might not be homologous. An independent origin of wing scales was recently documented in the now extinct lineage Kalligrammatidae (Neuroptera) (38), and body-covering scales are present in distantly related clades, such as spiders (Chelicerata) (101), springtails (Collembola), and archaeognathans (15, 95) (**Figure 3*b***). Alternatively, if the scale GRN evolved once in some ancestral arthropod and was maintained throughout evolution via its continuous expression in some part of the body, until the GRN became deployed on the wings in Amphiesmenoptera and Kalligrammatidae, then scales might be homologous. Scale GRN deployment on the wings would be analogous to endite GRN deployment on the abdomen to create prolegs in Lepidoptera. Future work should more closely map the evolution of scales across arthropods, as well as investigate the genetic basis of the GRNs of these multiple scales (similar to sense organ subtype identity analysis in beetles) (36) to test for scale GRN homology.

ORIGIN AND DEVELOPMENT OF EYESPOTS: CO-OPTION AND MODIFICATION OF A LIMB GRN

With the evolution of scales on the wing, and the diversification of the scale GRN into multiple scale morphologies and colors, lepidopterans began to organize these different colored scales into precise patterns on the wing, creating a variety of pattern innovations, among which are eyespots, which consist of concentric rings of colored scales (7, 54).

Recent work suggests that eyespots evolved independently in multiple members of the Lepidoptera (31). Early work, focusing exclusively on nymphalid butterflies, showed that eyespots originated once within this family, approximately 75 million years ago (65, 66). More recent work that scored and remapped the presence and absence of eyespots in a larger group of 715 species, representing almost all lineages of Lepidoptera, proposed that eyespots originated multiple times independently (31). Eyespots at positions similar to those of the nymphalid eyespots, for example, at the wing margin and in between wing veins, originated approximately 21 times independently, whereas those positioned more centrally on the wing, at or within the discal cell, originated approximately 30 times independently (**Figure 4*a***). This profusion of origins is the result of statistical analyses that estimated that a single origin of eyespots at the base of all moth lineages with eyespots is significantly less likely than multiple origins. From a developmental perspective, however, the independent origins of a similar pattern element (on a phylogeny) suggest that perhaps a shared and old GRN can be easily activated and deactivated on the wing.

Most studies of eyespot development have focused on nymphalid butterflies (reviewed in 7, 8, 14, 54), and very little is still known about moth eyespots (57) and eyespots that develop in other

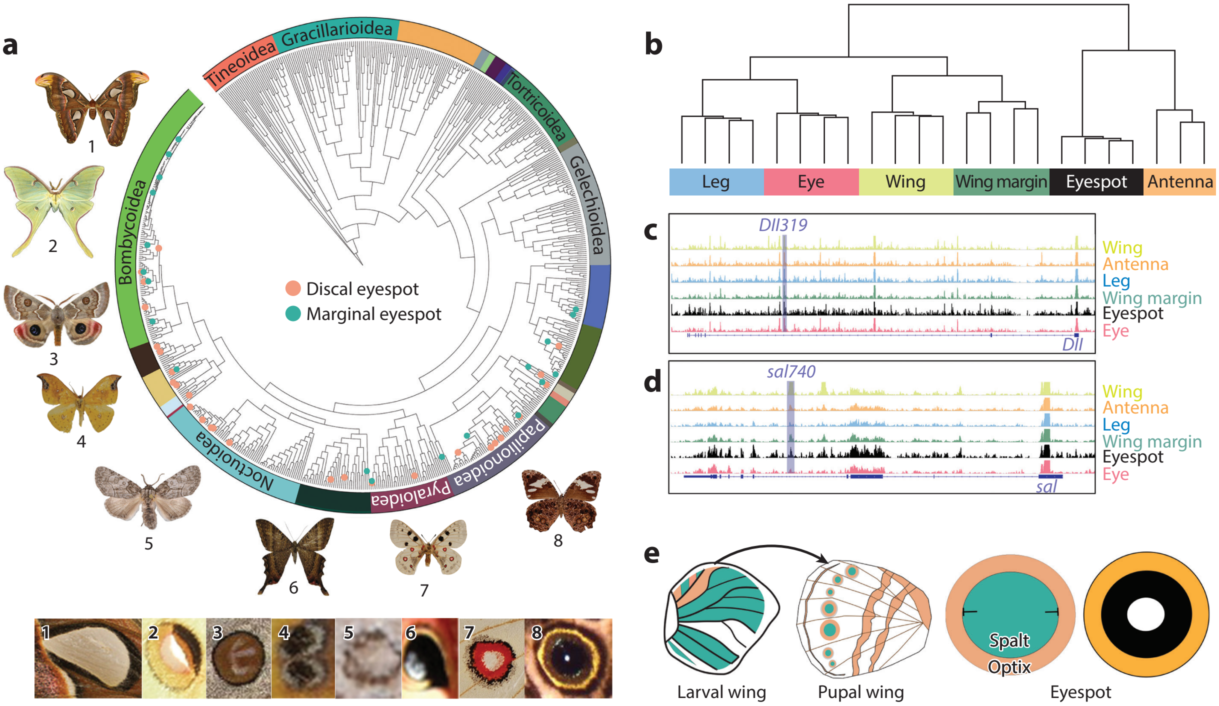


Figure 4

Phylogenetic and developmental origin of lepidopteran eyespots. (*a*) Phylogeny of Lepidoptera showing the independent and multiple origins of discal and marginal eyespots. (*b*) Hierarchical clustering of transcriptomes from different tissues collected at 3 h after pupation. The dissected eyespot tissue clusters first with antenna and then to other tissues. (*c*,*d*) Two pleiotropic enhancers of *Dll* and *sal* open across different tissues at 3 h after pupation identified by an assay for transposase-accessible chromatin with sequencing (ATAC-Seq). (*e*) Summary of the venation GRN from the larval stage co-option to the eyespot ring formation at the pupal stage. Abbreviations: *Dll*, *Distal-less*; GRN, gene-regulatory network; *sal*, *spalt*. Panel *a* based on data from Reference 31. Credits for images 1–8 of panel *a* are as follows: Image 1 (*Attacus atlas*) reproduced from the Smithsonian Institution (**https://collections.si.edu/search/results.htm?q=ark:/65665/3188d10cf-0b0c-467c-b710-c43d15f90b5b**) (CC0 1.0). Image 2 (*Actias luna*) adapted from James St. John [**https://commons.wikimedia.org/wiki/File:Actias_luna_(luna_moth)_(17257003162).jpg**] (CC BY 2.0). Image 3 (*Carthaea saturnioides*) reproduced with permission; copyright Dr. Stefan Naumann. Image 4 (*Tridrepana unispina*) modified from Guest of HoHuanShan/Flickr (**https://flickr.com/photos/44837798@N00/36260664306**) (CC BY 2.0). Image 5 (*Raphia abrupta*) reproduced from the Mississippi Entomological Museum (**https://www.gbif.org/occurrence/1838869103**) (CC0 1.0). Image 6 (*Sematura lunus*) from **https://commons.wikimedia.org/wiki/File:Sematura_lunus.JPG** (public domain). Image 7 (*Parnassius apollo*) reproduced from **https://lepdata.org/photos/animals/15223.jpg** (CC0 1.0). Image 8 (*Manataria hercyna*) reproduced from **https://lepdata.org/photos/animals/14207.jpg** (CC0 1.0). Panel *b* adapted from Reference 61. Panels *c* and *d* reproduced from Reference 60 (CC BY-NC-ND 4.0). Panel *e* adapted from Reference 4.

butterfly lineages, such as papilionids (80). However, a series of recent studies identified from where the nymphalid eyespot GRN may have derived, and these studies should be repeated in members of other lineages with eyespots. The studies confirmed that some of the genes expressed in nymphalid eyespots are connected in a network; i.e., the disruption of one leads to the disruption of expression of the others (50, 60). Further, two transcriptomic studies comparing genes expressed in the eyespot and adjacent control tissues in *Bicyclus anynana* butterflies identified approximately 800 differentially expressed genes within the center of the eyespot at the early pupal stage (3–6 h after pupation) (60, 68). The results indicate that a complex GRN underlies eyespot development and that the eyespot transcriptome was most similar to the antenna transcriptome, suggesting that a limb GRN was co-opted for eyespot development (60) (**Figure 4*b***).

To further test this idea, Murugesan et al. (60) identified CREs of two genes that are essential for both eyespot and limb development (*Dll* and *spalt*) and disrupted them with clustered regularly interspaced short palindromic repeats (CRISPR) (**Figure 4*c*,*d***). Mutations led to disruptions in limbs, wings, and eyespots, confirming that both *Dll* and *sal* were being driven by the same CRE in the two developmental contexts. This finding indicates that an ancestral GRN that patterns antennae, legs, and wings has been repurposed to pattern eyespots in nymphalid butterflies (56, 58, 61).

While a limb GRN co-option appears to underlie the differentiation of the eyespot central cells during the larval or early pupal stage, the differentiation of the different colored rings around the central cells appears to rely on a different GRN co-option event. Here, a preexisting vein patterning GRN that relies on a central morphogen gradient (of Decapentaplegic) to establish the position of veins at precise distances from the source (4) appears to have been co-opted to pattern the color rings. The similarity of genetic interactions between the processes of vein and color ring differentiation, in particular the cross-regulatory interactions between *optix* and *spalt* (3, 4, 9), suggests that both traits are using the same GRN (**Figure 4*e***).

Despite shared GRN elements, eyespots appear to be novel traits distinct from both limbs and veins. The co-option of the limb GRNs to the larval and pupal wing did not result in the formation of an appendage-like protrusion in the wing, and the co-option of the venation GRN to the pupal wing did not lead to extra "circular" veins being produced in pupal wings. At least three possibilities, which should be addressed by future work, could explain this outcome: (*a*) When a GRN is activated in a new developmental context (e.g., in a different tissue or in the same tissue but at a distinct stage of development), critical repressor or promoter proteins might be missing in that new context for the GRN to produce the exact same output. (*b*) Patterns of open chromatin, which determine which genes in the GRN become accessible for regulation, might also be different in the new contexts. (*c*) A partial, rather than a full, GRN co-option might have taken place. This could happen via the recruitment of genes in the middle of the GRN that are sufficient to activate some but not all the targets of the original GRN, leading to a different output and a different trait.

The multiple independent origins of eyespots across the Lepidoptera provide an excellent system to investigate the extent to which the GRN identified for nymphalid eyespots also underlies eyespot development in moth lineages (57). Its reuse across multiple lineages would indicate the highly modular and co-optable nature of this GRN. The use of new GRNs would, on the other hand, indicate that eyespots can be differentiated via the co-option of distinct GRNs. Alternatively, a single eyespot GRN combo (center + rings) originated once in a lineage sister to the Gelechioidea and has been repeatedly activated and deactivated throughout evolution and at different places in the wing.

ORIGIN OF BANDING PATTERNS IN BUTTERFLIES: BACKGROUND COLORS VERSUS BANDS

The emergence of scales enveloping the wing surface, coupled with a spatial coordinate system of differentiating scales of specific colors along transversal bands on the wing, fueled wing pattern novelties within the Lepidoptera. Most nymphalid butterflies share a system of bands typically known as the central symmetry system, the basal symmetry system, and the marginal bands, which together with the border ocelli form the nymphalid ground plan (63, 67). While a common origin for these bands is likely, the timing of the appearance of each band in the evolution of Lepidoptera remains poorly understood (75). Recent work, however, has elucidated some of the developmental architecture underlying these color bands. Banding pattern variations seem to depend on (*a*) genes that regulate the presence/absence of specific bands, (*b*) genes for band width and position, and (*c*) genes for background colors that appear in between the symmetry system bands.

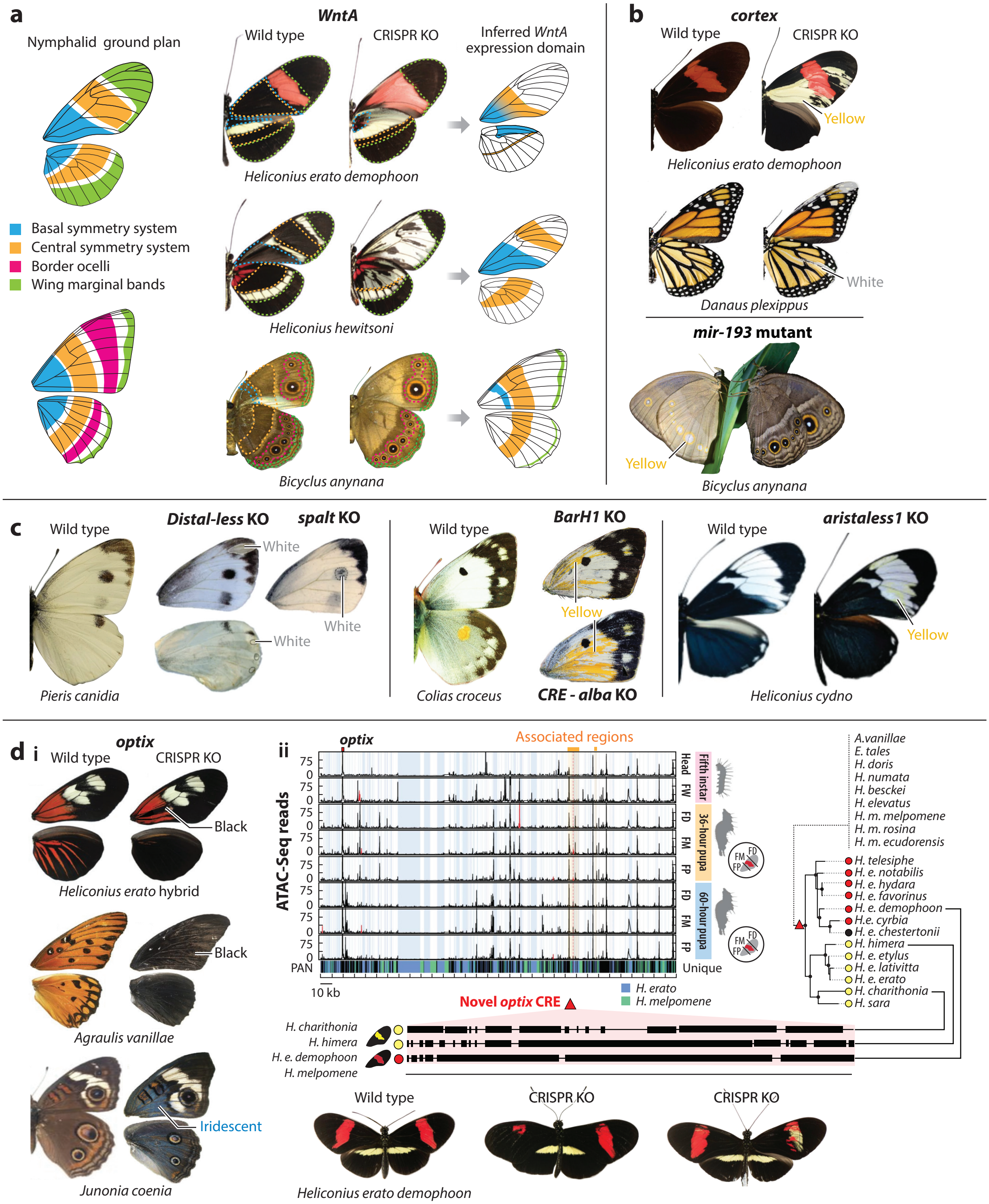
The *WntA* signaling molecule plays a crucial role in shaping the width (and coloring) of the basal and central symmetry systems of bands in nymphalids (5, 13, 24, 47, 48, 52). *WntA* is already expressed in domains that predict the adult wing pattern in larval wings and perfectly matches the adult phenotype in pupal wings (5). CRISPR disruptions of *WntA* affect the width and presence of these bands in various nymphalid species (5, 52), allowing background color to emerge in the form of white, yellow, or red bands depending on the species (13) (**Figure 5**).

A complex ~900-kb genomic region around the *cortex* locus seems to control melanism in scales. Mutations in this locus, induced by CRISPR or identified through mapping studies, often result in the transformation of dark melanic areas to yellow, white, or lighter colors (45, 62, 88) (**Figure 5**). This genomic region houses a long noncoding RNA (21, 44), which is a precursor to two highly conserved microRNAs (miRNAs), and all three are coexpressed in melanic wing regions of *B. anynana* (88). Mutations in one of the miRNAs (*mir-193*) produced light wing colors on the wing likely because this miRNA is a direct repressor of *ebony*, which shunts melanin precursors away from melanin pigment production (6, 49, 88). The observed broad spectrum of wing color mutations associated with this *cortex* locus suggests (*a*) the presence of a complex *cis*-regulatory architecture for the noncoding RNA and/or the miRNA or (*b*) the presence of complex genetic backgrounds interacting with alleles at this locus. This is evident in the polymorphic *Heliconius numata*, in which variations in yellow, white, black, and orange elements all map to this locus (33).

Two other genes, *aristaless1* and *optix*, not only promote specific background scale colors but also act as pigmentation switch genes. The homeobox gene *aristaless1* appears to promote white scales, with knockouts resulting in the transformation of white to yellow scales (99). Phylogenetic mapping of *aristaless1* haplotypes in *Heliconius* butterflies suggests the ancestral color state is yellow, with the appearance of white coloration in *H. cydno* and subsequent spread through hybridizations. *optix* has emerged as a master regulator of ommochrome pigments (72) but also as a suppressor of melanic pigmentation (106). Recent models for *optix* regulation propose that this gene can be expressed across most of the wing blade, but with TFs that bind a diverse set of *optix* CREs repressing it from areas that become black (40). In *H. erato demophoon*, *WntA* knockout results in red background color appearing in the bands of the basal and central symmetry systems, suggesting that *WntA* is a possible *optix* repressor (13) (**Figure 5*a***). However, when *optix* is repressed, the color black also appears (40, 106), suggesting a potential negative cross-regulation between *optix* and *WntA*.

Both *optix* and *WntA* have complex regulatory architectures and evolutionary histories composed of shared and novel lineage-specific CREs (53, 59, 92). Notably, these studies have revealed the evolution of a novel *H. erato* enhancer required to produce the red background color in *H. e. demophoon*, which implies a completely distinct architecture in the red co-mimic pattern of *Heliconius melpomene* (72, 93). The identification of a novel genomic CRE involved in background color regulation has also been achieved for *Colias* butterflies (Pieridae). Here, the insertion of a transposable element downstream of the homeobox gene *BarH1* led to its expression in the white form of *Colias crocea*. This novel expression of *BarH1* repressed pteridine pigment granules that appear in the yellow/orange forms of the same species (90, 102).

Recent studies have also identified two TFs, encoded by *Dll* and *spalt*, playing a role in marginal bands, chevrons, and wing tip color differentiation in nymphalid and *Pieris* butterflies. When these genes are disrupted, the dark marginal bands, chevrons, and black wing tips acquire the background color (14, 73, 98). The repression of *optix* by Spalt (observed in veins and in eyespot rings) (4) (**Figure 4*e***) might also be present in *Heliconius* wings and might be setting the distal edge



(Caption appears on following page)

Figure 5 (*Figure appears on preceding page*)

Genes and CREs that alter banding patterns and background colors in butterflies. Genes confirmed via CRISPR disruptions to function in patterning and coloring butterfly wings are shown. (*a*) The main patterning gene *WntA* controls the development of the basal and central symmetry systems in most nymphalids and the wing marginal bands in some (5, 13). These bands can significantly change in size and location depending on the species. (*b*) *Cis*-regulatory elements surrounding *cortex* and the effector gene *mir-193* in that locus, encoded by the lnc-RNA *ivory*, regulate melanic coloration (45, 88). (*c*) *spalt* and *Distal-less* regulate melanic marginal bands (which include distal spots) (98); *BarH1*, specifically driven by an identified CRE, and *aristaless1* promote white and suppress yellow across butterflies (90, 99, 102). (*d*, *i*) *optix* regulates ommochrome pigment production and represses melanin production; it also affects scale morphology (iridescence). These wing color pattern genes are controlled by complex regulatory architectures that include novel, lineage, or species-specific CREs, such as the one shown for *BarH1* (*alba*) (90) (panel *c*) and *optix* (93). (*d*, *ii*) The location of a novel *optix* CRE in the genome of *Heliconius erato demophoon* that controls a red-yellow background color switch (*orange vertical bar*) was mapped with the aid of ATAC-Seq data (93). This CRE is present in the *H. erato* clade but absent in the other *Heliconius* and outgroup species (*dotted lines*). The phylogeny represented corresponds to the CRE phylogeny. Abbreviations: ATAC-Seq, assay for transposase-accessible chromatin with sequencing; CRE, *cis*-regulatory element; CRISPR, clustered regularly interspaced short palindromic repeats; KO, knockout. In panel *a*, images of *H. erato demophoon* and *H. hewitsoni* are reproduced from Reference 13 and images of *Bicyclus anynana* are reproduced from Reference 5 (CC BY 4.0). In panel *b*, images of *H. erato demophoon* and *Danaus plexippus* are reproduced from Reference 45 (CC BY 4.0); images of *B. anynana* are reproduced with permission; copyright Shen Tian. In panel *c*, images of *Pieris canidia* are adapted from Reference 98 (CC BY 4.0), left and top-right images of *Colias croceus* are adapted from Reference 102 (CC BY 4.0) and bottom-right image is adapted from Reference 90 (CC BY 4.0), and images of *Heliconius cydno* are adapted from Reference 99; copyright Elsevier. In panel *d*, subpanel *i*, images of *H. erato*, *Agraulis vanillae*, and *Junonia coenia* are reproduced with permission from Reference 106 and subpanel *ii* is adapted from Reference 93.

of *optix* expression in these species. Future work should characterize the GRN architecture and connection of all these fundamental patterning and coloring genes. Addressing such interactions during the pupal developmental stage is especially important to better comprehend how final pigmentation at the adult stage is determined.

CONCLUSIONS AND FUTURE DIRECTIONS

Lepidopterans are excellent models to examine the evolution of novel traits such as prolegs, different colored wing scales, eyespots, and banding patterns. All these traits appear to involve the use of preexisting GRNs in novel ways. The GRNs control units of interconnected genes that can be expressed, in whole or in part, in new contexts. The new context, in turn, can alter the output of the GRN to produce a different trait rather than a repeated trait. In this review we presented (*a*) a GRN that controls endite growth, co-opted to produce prolegs; (*b*) a GRN that differentiates sensory bristles, co-opted to the wing and modified to produce scales; (*c*) a GRN that controls limb outgrowth, co-opted to differentiate eyespot centers of nymphalid butterflies; (*d*) a GRN that controls wing venation, co-opted to differentiate the rings of color in nymphalid butterfly eyespots; and (*e*) GRNs that control the suite of metabolic genes involved in melanin, ommochrome, and pteridin pigment production that are regulated by specific switches (CREs) placed at genes such as *WntA*, *optix*, or *BarH1*.

Future work is required to understand how the co-option of GRNs leads to novel traits rather than just repeated (serial homologous) traits (**Figure 1**). While current work is dissecting the CREs that are involved in the origin and regulation of some of these co-option events, future work is required to examine in greater detail the regulatory interactions that are taking place between the causative genetic mutations and the GRNs, which might also determine the extent to which, and at what level, the GRN was co-opted. This type of investigation can involve knockout experiments in focal genes followed by an investigation of which other genes were affected and are, thus, downstream of the focal target. The identification of pleiotropic CREs of genes that regulate old and new traits (as done for *Dll* and *spalt* in the context of limbs and eyespots) helps identify what (parts of) networks are being shared among traits. Likewise, the identification of nonpleiotropic and novel CREs (of *optix* in *Heliconius* spp. and of *BarH1* in

Colias spp.) helps identify points of GRN divergence and how novel traits acquire their own identities.

The future deployment of single-cell sequencing, chromatin accessibility, and spatial transcriptomics, combined with functional tools, is likely to continue to aid our understanding of how novel complex traits, which evolve from preexisting GRNs, come to acquire their unique identities over the course of evolution. Specifically, the ability to discover similar cell types in distinct parts of the body and in complex tissues with similar chromatin and gene expression profiles may reveal instances of GRN co-option that have, so far, been unacknowledged. This fine-grained examination of gene expression similarity at the single-cell level will also help us understand how new cell types arise in evolution. Finally, artificial intelligence is likely to become essential in the future to delineate and identify modules, or GRNs, that lend themselves to co-option.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

Research was supported by the National Research Foundation (NRF), Singapore, Investigatorship award (NRF-NRFI05-2019-0006, NRF-CRP awards (NRF-CRP20-2017-0001 and NRF-CRP25-2020-0001), and Ministry of Education (MOE), Singapore award (MOE-T2EP30222-0017) (to A.M.); by a Human Frontier Science Program Research Grant (RGP0034/2021) (A.P.); and by EPSCoR RII Track-2 FEC (OIA 1736026), by the Puerto Rico Science, Technology and Research Trust (ARG 2020-00138 & CRG 2020-00142), and by the Hispanic Alliance for Clinical and Translational Research (Alliance) supported by the National Institute of General Medical Sciences (NIGMS), National Institutes of Health (U54GM133807) and the P20 GM103475 from NIGMS, a component of the NIH INBRE (to R.P.).

LITERATURE CITED

1. Ansari S, Troelenberg N, Dao VA, Richter T, Bucher G, Klingler M. 2018. Double abdomen in a short-germ insect: zygotic control of axis formation revealed in the beetle *Tribolium castaneum*. *PNAS* 115(8):1819–24
2. Banerjee TD, Monteiro A. 2018. CRISPR-Cas9 mediated genome editing in *Bicyclus anynana* butterflies. *Methods Protoc.* 1:16
3. Banerjee TD, Monteiro A. 2020. Molecular mechanisms underlying simplification of venation patterns in holometabolous insects. *Development* 147:196394
4. Banerjee TD, Monteiro A. 2021. Reuse of an insect wing venation gene-regulatory subnetwork in patterning the eyespot rings of butterflies. bioRxiv 2021.05.22.445259. **https://doi.org/10.1101/2021.05.22.445259**
5. Banerjee TD, Murugesan SN, Connahs H, Monteiro A. 2023. Spatial and temporal regulation of Wnt signaling pathway members in the development of butterfly wing patterns. *Sci. Adv.* 9:eadg3877
6. Barek H, Veraksa A, Sugumaran M. 2018. *Drosophila melanogaster* has the enzymatic machinery to make the melanic component of neuromelanin. *Pigment Cell Melanoma Res.* 31:683–92
7. Beldade P, Monteiro A. 2021. Eco-evo-devo advances with butterfly eyespots. *Curr. Opin. Genet. Dev.* 69:6–13
8. Beldade P, Peralta CM. 2017. Developmental and evolutionary mechanisms shaping butterfly eyespots. *Curr. Opin. Insect Sci.* 19:22–29
9. Bier E. 2000. Drawing lines in the *Drosophila* wing: initiation of wing vein development. *Curr. Opin. Genet. Dev.* 10:393–98

10. Bitsch J. 2012. The controversial origin of the abdominal appendage-like processes in immature insects: Are they true segmental appendages or secondary outgrowths? (Arthropoda Hexapoda). *J. Morphol.* 273:919–31
11. Blochlinger K, Bodmer R, Jack J, Jan LY, Jan YN. 1988. Primary structure and expression of a product from *cut*, a locus involved in specifying sensory organ identity in *Drosophila*. *Nature* 333:629–35
12. Challi RJ, Kumar S, Dasmahapatra KK, Jiggins CD, Blaxter M. 2016. Lepbase: the lepidopteran genome database. bioRxiv 056994. **https://doi.org/10.1101/056994**
13. Concha C, Wallbank RW, Hanly JJ, Fenner J, Livraghi L, et al. 2019. Interplay between developmental flexibility and determinism in the evolution of mimetic *Heliconius* wing patterns. *Curr. Biol.* 29:3996–4009.e4
14. Connahs H, Tlili S, van Creij J, Loo TYJ, Banerjee TD, et al. 2019. Activation of butterfly eyespots by Distal-less is consistent with a reaction-diffusion process. *Development* 146:dev169367
15. D'Alba L, Wang B, Vanthournout B, Shawkey MD. 2019. The golden age of arthropods: ancient mechanisms of colour production in body scales. *J. R. Soc. Interface* 16:20190366
16. Darwin Tree of Life Consort. 2022. Sequence locally, think globally: the Darwin Tree of Life Project. *PNAS* 119:e2115642118
17. Day CR, Hanly JJ, Ren A, Martin A. 2019. Sub-micrometer insights into the cytoskeletal dynamics and ultrastructural diversity of butterfly wing scales. *Dev. Dyn.* 248:657–70
18. DiFrisco J, Love AC, Wagner GP. 2023. The hierarchical basis of serial homology and evolutionary novelty. *J. Morphol.* 284:e21531
19. Dinwiddie A, Null R, Pizzano M, Chuong L, Krup AL, et al. 2014. Dynamics of F-actin prefigure the structure of butterfly wing scales. *Dev. Biol.* 392:404–18
20. Emerson MJ, Schram FR. 1991. Remipedia. Part 2: paleontology. *Proc. San Diego Soc. Nat. Hist.* 7:1–52
21. Fandino RA, Brady NK, Chatterjee M, McDonald JM, Livraghi L, et al. 2024. The *ivory* lncRNA regulates seasonal color patterns in buckeye butterflies. bioRxiv 2024.02.09.579733. **https://doi.org/10.1101/2024.02.09.579733**
22. Fusari LM, Dantas GPS, Pinho LC. 2018. Order Diptera. In *Thorp and Covich's Freshwater Invertebrates*, Volume 3: *Keys to Neotropical Hexapoda*, ed. N Hamada, JH Thorp, DC Rogers, pp. 607–23. Amsterdam: Elsevier. 4th ed.
23. Galant R, Skeath JB, Paddock S, Lewis DL, Carroll SB. 1998. Expression pattern of a butterfly *achaete-scute* homolog reveals the homology of butterfly wing scales and insect sensory bristles. *Curr. Biol.* 8:807–13
24. Gallant JR, Imhoff VE, Martin A, Savage WK, Chamberlain NL, et al. 2014. Ancient homology underlies adaptive mimetic diversity across butterflies. *Nat. Commun.* 5:4817
25. Gallicchio L, Griffiths-Jones S, Ronshaugen M. 2021. Single-cell visualization of *mir-9a* and *Senseless* co-expression during *Drosophila melanogaster* embryonic and larval peripheral nervous system development. *G3* 11:jkaa010
26. Ghiradella H. 1989. Structure and development of iridescent butterfly scales: lattices and laminae. *J. Morphol.* 202:69–88
27. Ghiradella H. 1994. Structure of butterfly scales: patterning in an insect cuticle. *Microsc. Res. Tech.* 27:429–38
28. Giribet G, Edgecombe GD. 2019. The phylogeny and evolutionary history of arthropods. *Curr. Biol.* 29:R592–602
29. Grimaldi D, Engel MS. 2005. *Evolution of the Insects*. Cambridge, UK: Cambridge Univ. Press
30. Hanly JJ, Francescutti CM, Loh LS, Corning OB, Long DJ, et al. 2023. Genetics of yellow-orange color variation in a pair of sympatric sulfur butterflies. *Cell Rep.* 42:112820
31. Hanotte B, Willink B, Monteiro A. 2024. Eyespots originated multiple times independently across the Lepidoptera. bioRxiv 2024.02.07.579046. **https://doi.org/10.1101/2024.02.07.579046**
32. Hinton HE. 1955. On the structure, function, and distribution of the prolegs of the Panorpoidea, with a criticism of the Berlese-Imms theory. *Trans. R. Entomol. Soc. London* 106:455–540
33. Joron M, Frezal L, Jones RT, Chamberlain NL, Lee SF, et al. 2011. Chromosomal rearrangements maintain a polymorphic supergene controlling butterfly mimicry. *Nature* 477:203–6

34. Kavaler J, Fu W, Duan H, Noll M, Posakony JW. 1999. An essential role for the *Drosophila Pax2* homolog in the differentiation of adult sensory organs. *Development* 126:2261–72
35. Kilchoer C, Steiner U, Wilts BD. 2019. Thin-film structural coloration from simple fused scales in moths. *J. R. Soc. Interface Focus* 9:20180044
36. Klann M, Schacht MI, Benton MA, Stollewerk A. 2021. Functional analysis of sense organ specification in the *Tribolium castaneum* larva reveals divergent mechanisms in insects. *BMC Biol*. 19:21
37. Kou L-X, Hua B-Z. 2016. Comparative embryogenesis of Mecoptera and Lepidoptera with special reference to the abdominal prolegs. *J. Morphol.* 277:585–93
38. Labandeira CC, Yang Q, Santiago-Blay JA, Hotton CL, Monteiro A, et al. 2016. The evolutionary convergence of mid-Mesozoic lacewings and Cenozoic butterflies. *Proc. R. Soc. B* 283:20152893
39. Lewis DL, DeCamillis M, Bennett RL. 2000. Distinct roles of the homeotic genes Ubx and abd-A in beetle embryonic abdominal appendage development. *PNAS* 97:4504–9
40. Lewis JJ, Geltman RC, Pollak PC, Rondem KE, Van Belleghem SM, et al. 2019. Parallel evolution of ancient, pleiotropic enhancers underlies butterfly wing pattern mimicry. *PNAS* 116:24174–83
41. Li J-J, Shi Y, Wu J-N, Li H, Smagghe G, Liu T-X. 2021. CRISPR/Cas9 in lepidopteran insects: progress, application and prospects. *J. Insect Physiol.* 135:104325
42. Liu B-P, Hua B-Z. 2024. Distinct roles of the Hox genes *Ultrabithorax* and *abdominal-A* in scorpionfly embryonic proleg development. *Insect Mol. Biol.* 33:69–80
43. Liu J, Chen Z, Xiao Y, Asano T, Li S, et al. 2021. Lepidopteran wing scales contain abundant cross-linked film-forming histidine-rich cuticular proteins. *Commun. Biol.* 4:491
44. Livraghi L, Hanly JJ, Evans E, Wright CJ, Loh LS, et al. 2024. A long non-coding RNA at the *cortex* locus controls adaptive colouration in butterflies. bioRxiv 2024.02.09.579710. **https://doi.org/10.1101/2024.02.09.579710**
45. Livraghi L, Hanly JJ, Van Bellghem SM, Montejo-Kovacevich G, van der Heijden ESM, et al. 2021. *Cortex cis*-regulatory switches establish scale colour identity and pattern diversity in *Heliconius*. *eLife* 10:e68549
46. Lloyd VJ, Burg SL, Harizanova J, Hill O, Enciso-Romero J, et al. 2023. The actin cytoskeleton plays multiple roles in structural color formation in butterfly wing scales. bioRxiv 2023.06.01.542791. **https://doi.org/10.1101/2023.06.01.542791**
47. Martin A, Papa R, Nadeau NJ, Hill RI, Counterman BA, et al. 2012. Diversification of complex butterfly wing patterns by repeated regulatory evolution of a *Wnt* ligand. *PNAS* 109:12632–37
48. Martin A, Reed RD. 2014. *Wnt* signaling underlies evolution and development of the butterfly wing pattern symmetry systems. *Dev. Biol.* 395:367–78
49. Matsuoka Y, Monteiro A. 2018. Melanin pathway genes regulate color and morphology of butterfly wing scales. *Cell Rep*. 24:56–65
50. Matsuoka Y, Monteiro A. 2022. *Ultrabithorax* modifies a regulatory network of genes essential for butterfly eyespot development in a wing sector-specific manner. *Development* 149:dev200781
51. Matsuoka Y, Murugesan SN, Prakash A, Monteiro A. 2023. Lepidopteran prolegs are novel traits, not leg homologs. *Sci. Adv.* 9:eadd9389
52. Mazo-Vargas A, Concha C, Livraghi L, Massardo D, Wallbank RW, et al. 2017. Macroevolutionary shifts of *WntA* function potentiate butterfly wing-pattern diversity. *PNAS* 114:10701–6
53. Mazo-Vargas A, Langmüller AM, Wilder A, van der Burg KR, Lewis JJ, et al. 2022. Deep *cis*-regulatory homology of the butterfly wing pattern ground plan. *Science* 378:304–8
54. Monteiro A. 2015. Origin, development, and evolution of butterfly eyespots. *Annu. Rev. Entomol.* 60:253–71
55. Monteiro A. 2021. Distinguishing serial homologs from novel traits: experimental limitations and ideas for improvements. *Bioessays* 43:2000162
56. Monteiro A, Das Gupta M. 2016. Identifying co-opted networks and causative mutations in the origin of novel complex traits. *Curr. Top. Dev. Biol.* 119:205–26
57. Monteiro A, Glaser G, Stockslagger S, Glansdorp N, Ramos DM. 2006. Comparative insights into questions of lepidopteran wing pattern homology. *BMC Dev. Biol.* 6:52

58. Monteiro A, Podlaha O. 2009. Wings, horns, and butterfly eyespots: How do complex traits evolve? *PLOS Biol*. 7:209–16
59. Morris J, Navarro N, Rastas P, Rawlins LD, Sammy J, et al. 2019. The genetic architecture of adaptation: convergence and pleiotropy in *Heliconius* wing pattern evolution. *Heredity* 123:138–52
60. Murugesan SN, Connahs H, Matsuoka Y, Das Gupta M, Tiong GJL, et al. 2022. Butterfly eyespots evolved via cooption of an ancestral gene-regulatory network that also patterns antennae, legs, and wings. *PNAS* 119:e2108661119
61. Murugesan SN, Monteiro A. 2022. Evolution of modular and pleiotropic enhancers. *J. Exp. Zool. B Mol. Dev. Evol.* 340(2):105–15
62. Nadeau NJ, Pardo-Diaz C, Whibley A, Supple MA, Saenko SV, et al. 2016. The gene *cortex* controls mimicry and crypsis in butterflies and moths. *Nature* 534:106–10
63. Nijhout HF. 1990. A comprehensive model for colour pattern formation in butterflies. *Proc. R. Soc. B* 239:81–113
64. Nolo R, Abbott LA, Bellen HJ. 2000. Senseless, a Zn finger transcription factor, is necessary and sufficient for sensory organ development in *Drosophila*. *Cell* 102:349–62
65. Oliver JC, Beaulieu JM, Gall LF, Piel WH, Monteiro A. 2014. Nymphalid eyespot serial homologs originate as a few individualized modules. *Proc. R. Soc. B* 281:20133262
66. Oliver JC, Tong X-L, Gall LF, Piel WH, Monteiro A. 2012. A single origin for nymphalid butterfly eyespots followed by widespread loss of associated gene expression. *PLOS Genet.* 8:e1002893
67. Otaki JM. 2012. Color pattern analysis of nymphalid butterfly wings: revision of the nymphalid groundplan. *Zool. Sci.* 29:568–76
68. Özsu N, Monteiro A. 2017. Wound healing, calcium signaling, and other novel pathways are associated with the formation of butterfly eyespots. *BMC Genom*. 18:788
69. Piszter G, Kertész K, Bálint Z, Biró LP. 2023. Wide-gamut structural colours on oakblue butterflies by naturally tuned photonic nanoarchitectures. *R. Soc. Open Sci.* 10:221487
70. Prakash A, Dion E, Monteiro A. 2023. The molecular basis of macrochaete diversification highlighted by a single-cell atlas of *Bicyclus anynana* butterfly pupal forewings. bioRxiv 2023.08.23.554425. **https://doi.org/10.1101/2023.08.23.554425**
71. Prakash A, Finet C, Banerjee TD, Saranathan V, Monteiro A. 2022. *Antennapedia* and *optix* regulate metallic silver wing scale development and cell shape in *Bicyclus anynana* butterflies. *Cell Rep*. 40:111052
72. Reed RD, Papa R, Martin A, Hines HM, Counterman BA, et al. 2011. *optix* drives the repeated convergent evolution of butterfly wing pattern mimicry. *Science* 333:1137–41
73. Reed RD, Selegue JE, Zhang L, Brunetti CR. 2020. Transcription factors underlying wing margin color patterns and pupal cuticle markings in butterflies. *EvoDevo* 11:10
74. Ren A, Day CR, Hanly JJ, Counterman BA, Morehouse NI, Martin A. 2020. Convergent evolution of broadband reflectors underlies metallic coloration in butterflies. *Front. Ecol. Evol.* 8:206
75. Schachat SR, Brown RL. 2016. Forewing color pattern in Micropterigidae (Insecta: Lepidoptera): homologies between contrast boundaries, and a revised hypothesis for the origin of symmetry systems. *BMC Evol. Biol.* 16:116
76. Schweisguth F. 2015. Asymmetric cell division in the *Drosophila* bristle lineage: from the polarization of sensory organ precursor cells to Notch-mediated binary fate decision. *Wiley Interdiscip. Rev. Dev. Biol.* 4:299–309
77. Scoble MJ. 1992. *The Lepidoptera: Form, Function and Diversity*. Oxford, UK: Oxford Univ. Press
78. Scourfield DJ. 1926. On a new type of crustacean from the Old Red Sandstone (Rhynie Chert Bed, Aberdeenshire)-*Lepidocaris rhyniensis* gen. et sp. nov. *Philos. Trans. R. Soc. B* 214:153–87
79. Seah KS, Saranathan V. 2023. Hierarchical morphogenesis of swallowtail butterfly wing scale nanostructures. *eLife* 12:RP89082
80. Shirai LT, Saenko SV, Keller RA, Jeronimo MA, Brakefield PM, et al. 2012. Evolutionary history of the recruitment of conserved developmental genes in association to the formation and diversification of a novel trait. *BMC Evol. Biol.* 12:21
81. Simonsen TJ. 2001. The wing vestiture of the non-ditrysian Lepidoptera (Insecta). Comparative morphology and phylogenetic implications. *Acta Zool*. 82:275–98

82. Snodgrass RE. 1931. Morphology of the insect abdomen. *Smithson. Misc. Collect.* 85(6):1–128
83. Snodgrass RE. 1993. *Principles of Insect Morphology*. Ithaca, NY: Cornell Univ. Press
84. Sundermann A, Lohse S, Beck L, Haase P. 2007. Key to the larval stages of aquatic true flies (Diptera), based on the operational taxa list for running waters in Germany. *Proc. Ann. Limnol.* 43:61–74
85. Suzuki Y, Palopoli MF. 2001. Evolution of insect abdominal appendages: Are prolegs homologous or convergent traits? *Dev. Genes Evol.* 211:486–92
86. Thayer RC, Patel NH. 2023. A meta-analysis of butterfly structural colors: their color range, distribution and biological production. *J. Exp. Biol.* 226:jeb245940
87. Thümecke S, Schröder R. 2022. The *odd-skipped* related gene *drumstick* is required for leg development in the beetle *Tribolium castaneum*. *Dev. Dyn.* 251:1456–71
88. Tian S, Asano Y, Banerjee TD, Wee JLQ, Lamb A, et al. 2024. A micro-RNA is the effector gene of a classic evolutionary hotspot locus. bioRxiv 2024.02.09.579741. **https://doi.org/10.1101/2024.02.09.579741**
89. True JR, Carroll SB. 2002. Gene co-option in physiological and morphological evolution. *Annu. Rev. Cell Dev. Biol.* 18:53–80
90. Tunström K, Woronik A, Hanly JJ, Rastas P, Chichvarkhin A, et al. 2023. Evidence for a single, ancient origin of a genus-wide alternative life history strategy. *Sci. Adv.* 9:eabq3713
91. Vachon G, Cohen B, Pfeifle C, McGuffin ME, Botas J, Cohen SM. 1992. Homeotic genes of the Bithorax complex repress limb development in the abdomen of the Drosophila embryo through the target gene *Distal-less*. *Cell* 71:437–50
92. Van Belleghem SM, Rastas P, Papanicolaou A, Martin SH, Arias CF, et al. 2017. Complex modular architecture around a simple toolkit of wing pattern genes. *Nat. Ecol. Evol.* 1:0052
93. Van Belleghem SM, Ruggieri AA, Concha C, Livraghi L, Hebberecht L, et al. 2023. High level of novelty under the hood of convergent evolution. *Science* 379:1043–49
94. van Eldijk TJ, Wappler T, Strother PK, van der Weijst CM, Rajaei H, et al. 2018. A Triassic-Jurassic window into the evolution of Lepidoptera. *Sci. Adv.* 4:e1701568
95. Vanthournout B, Rousaki A, Parmentier T, Janssens F, Mertens J, et al. 2021. Springtail coloration at a finer scale: mechanisms behind vibrant collembolan metallic colours. *J. R. Soc. Interface* 18:20210188
96. Wang J, Zhang W, Engel MS, Sheng X, Shih C, Ren D. 2022. Early evolution of wing scales prior to the rise of moths and butterflies. *Curr. Biol.* 32:3808–14.e2
97. Warren RW, Nagy L, Selegue J, Gates J, Carroll S. 1994. Evolution of homeotic gene regulation and function in flies and butterflies. *Nature* 372:458–61
98. Wee JLQ, Das Banerjee T, Prakash A, Seah KS, Monteiro A. 2022. *Distal-less* and *spalt* are distal organisers of pierid wing patterns. *EvoDevo* 13:12
99. Westerman EL, VanKuren NW, Massardo D, Tenger-Trolander A, Zhang W, et al. 2018. *Aristaless* controls butterfly wing color variation used in mimicry and mate choice. *Curr. Biol.* 28:3469–74.e4
100. Wilts BD, Apeleo Zubiri B, Klatt MA, Butz B, Fischer MG, et al. 2017. Butterfly gyroid nanostructures as a time-frozen glimpse of intracellular membrane development. *Sci. Adv.* 3:e1603119
101. Wilts BD, Otto J, Stavenga DG. 2020. Ultra-dense, curved, grating optics determines peacock spider coloration. *Nanoscale Adv.* 2:1122–27
102. Woronik A, Tunström K, Perry MW, Neethiraj R, Stefanescu C, et al. 2019. A transposable element insertion is associated with an alternative life history strategy. *Nat. Commun.* 10:5757
103. Wright CJ, Stevens L, Mackintosh A, Lawniczak M, Blaxter M. 2024. Comparative genomics reveals the dynamics of chromosome evolution in Lepidoptera. *Nat. Ecol. Evol.* 8(4):777–90
104. Yue C, Hua B. 2010. Are abdominal prolegs serially homologous with the thoracic legs in Panorpidae (Insecta: Mecoptera)? Embryological evidence. *J. Morphol.* 271:1366–73
105. Zhang F, Chen Z, Dong R-R, Deharveng L, Stevens MI, et al. 2014. Molecular phylogeny reveals independent origins of body scales in Entomobryidae (Hexapoda: Collembola). *Mol. Phylogenet. Evol.* 70:231–39
106. Zhang L, Mazo-Vargas A, Reed RD. 2017. Single master regulatory gene coordinates the evolution and development of butterfly color and iridescence. *PNAS* 114:10707–12

107. Zhang L, Reed RD. 2017. A practical guide to CRISPR/Cas9 genome editing in Lepidoptera. In *Diversity and Evolution of Butterfly Wing Patterns: An Integrative Approach*, ed. T Sekimura, HF Nijhout, pp. 155–72. Singapore: Springer

108. Zhang Q, Mey W, Ansorge J, Starkey TA, McDonald LT, et al. 2018. Fossil scales illuminate the early evolution of lepidopterans and structural colors. *Sci. Adv.* 4:e1700988

109. Zhao Y-G, Hua B-Z. 2016. Morphology of the immature stages of *Arge pagana* (Panzer, 1798) (Hymenoptera: Argidae) with notes on its biology. *J. Asia-Pac. Entomol.* 19:903–9