**Cellular and Developmental Basis of Avian Structural Coloration**

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Vivid structural colors in birds are a conspicuous and vital part of their phenotype. They are produced by a rich diversity of integumentary photonic nanostructures in skin and feathers. Unlike pigmentary coloration, whose molecular genetic basis is being elucidated, little is known regarding the pathways underpinning organismal structural coloration. Here, we review available data on the development of avian structural colors. In particular, feather photonic nanostructures are understood to be intracellularly self-assembled by physicochemical forces typically seen in soft colloidal systems. We identify promising avenues for future research that can address current knowledge gaps, which is also highly relevant for the sustainable engineering of advanced bioinspired and biomimetic materials.

**Keywords:** structural colors, biophotonic nanostructures, self-assembly, skin coloration, plumage coloration

**INTRODUCTION**

While pigmentary colors result from wavelength-selective molecular absorption and re-emission of light, vivid saturated colors are produced via physical or structural means, or sometimes a combination of both [1, 2\textsuperscript{*}]. Organismal structural colors arise from light scattering by biophotonic nanostructures with compositional variation (i.e., refractive index contrast) on the order of visible light wavelengths [1, 3]. They can be further classified based on whether the scattering is incoherent (e.g., white color), arising from uncorrelated or spatially independent scatterers (in Rayleigh, Tyndall or Mie regimes depending on particle size) or coherent, as a result of constructive interference of light due to periodic or quasi-periodic spatial material variation with characteristic length scales of about 100-350 nm [1, 3]. The latter class of interference colors, especially (ultra)violet, blue and green hues are quite conspicuous in animals and produced by a stunning diversity of underlying epidermal or integumentary photonic nanostructures [1, 4, 5]. They constitute a very important aspect of the appearance of animals including birds, as they are often used in aposematism, crypsis or in inter- and intra-sexual signaling [2\textsuperscript{*}][6].

By contrast to pigment-based coloration and pattern formation, there is a dearth of developmental studies on organismal structural coloration in general, and their underlying genetic basis is only just unraveling [7\textsuperscript{**}, 8\textsuperscript{*}][9]. This is in part, because most model species lack structural coloration or remain uninvestigated, even if present. In this review, we describe the progress to date in understanding the genetics and development of structural color production in birds, a cosmopolitan group of over 10,000 species with vibrant and diverse coloration [4, 6]. We identify promising avenues for future research that can address current knowledge gaps.

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**STRUCTURAL COLORATION IN AVIAN SKIN**

Non-iridescent structural colors prominently occur in bare skin (especially around the eye), bill (ramphotheca) and feet (podotheca) and has convergently evolved in over 50 bird families, likely driven by sexual selection [10, 11\textsuperscript{**}] (Figs. 1A-B). They are produced by constructive light interference from 2D quasi-periodic arrays of parallel collagen (Refractive Index 1.42) fibrils in the mucopolysaccharide matrix (RI 1.35) of the dermis and underlain by a layer of melanin granules called melanosomes [10, 11\textsuperscript{**}] (Fig. 2A). The collagen fibrils are in turn bundled into larger macrofibrils (fibers), tens of microns in diameter and several hundred microns in length that are apparently produced by a single collagenocyte in such a way that their longitudinal axis is aligned nearly parallel to the skin surface [10]. However, the Velvet Asity (Philepitta castanea) uniquely among birds has evolved a 2D photonic crystal (PC) [3] analog with ordered hexagonal arrangement of collagen fibrils that is derived from ancestral quasi-periodic state found in the sister sunbird asities (Neodrepanis spp.) [10, 12]. This transition is apparently driven by female preference for highly saturated hues in this lek polygynous species rather than for directional optical properties, as the overall papillose geometry of the facial caruncle and the polycrystalline nature of macrofibrils attenuate iridescence [10, 12].

The genetic and developmental basis of structural color producing dermal collagen arrays remains unstudied. Nevertheless, Prum and coworkers [10] have suggested some prerequisites for the evolution of photonic dermal collagen arrays. In a plausible sequence, these include – loss of feathers exposing bare skin (apteria), thickening of dermis with a concomitant proliferation of collagen arrays to increase scattering efficiency given their low refractive index contrast, dermal melanization to absorb any unscattered light that would otherwise wash out structural hues, and near-uniform specification of larger than usual fibrils (> 100 nm) to make avian visible hues.
It is conceivable that evolution of bare skin in birds is accompanied by dermal thickening for mechanical reasons and melanization as protection against UV damage.

Here, we focus on the molecular basis of collagen fibrillogenesis, which ultimately determines the photonic aspect of dermal collagen arrays (Fig. 2A). The hierarchical assembly of triple-helical collagen proteins that occurs in the dermal extracellular matrix (ECM) into collagen fibres is intrinsic and integral to the structure and function of vertebrate connective tissues, including dermis [13]. Collagens, specified by the diverse COL gene family, are ubiquitous and largely conserved across vertebrates, although birds have a slightly reduced diversity [14]. The fact that collagen synthesis and fibrillogenesis in vertebrates are tightly regulated [13], with large irregularly sized fibrils seen pathologically [15], suggests that photonic organization of fibrillar bundles likely stems from molecular regulation of fibril diameter, inter-fibril spacing and arrangement. One promising regulator is Tenascin-X (TNX; Tenascin-Y in birds [16]) with epidermal growth factor (EGF) and fibronectin (FN) domains, which is implicated in determining inter-fibril spacing as well as accelerating rate of fibril formation by interacting with ECM proteoglycans such as decorin [13]. Interestingly, loss of either decorin [17] or TNX [18] results in irregularly arranged, large-diameter fibrils. The ETS family transcription factor FLI1 (with known avian homolog) is also of interest as it represses fibrillar collagen genes, while upregulating the production of small leucine-rich proteoglycan (including decorin), during fibrillogenesis [19]. However, spatio-temporal changes in fibrillar and fibril-associated collagen expression could result in similar changes in phenotype [15], suggesting that family-specific differences in collagen fibril composition could also be responsible for the repeated evolution of this trait in birds [14].
STRUCTURAL COLORATION IN AVIAN PLUMAGES

Iridescent Feather Barbule Coloration

Structural coloration in feather barbules is generally iridescent (Figs. 1C-D) and produced by interference from biological analogs of 1DPCs [3]—thin-film or multilayer (spaced or close-packed) arrays of melanosomes (RI ~ 2.0) embedded in a β-keratin medium (RI 1.58) [11**] (Fig. 2E). However, considerable systematic variation exists in the morphology and arrangement of melanosomes within iridescent barbules [See Fig. 3 of 20][21, 22]. The melanosomes can be spherical, lozenge to rod-shaped about 1-2 µm in length with various aspect ratios, or pancake-shaped. The melanosomes involved in barbule iridescence are usually comprised of eumelanin, although some pigeons (Columbia trocaz) utilize phaeomelanins [11**]. Some taxa (starlings, hummingbirds and quetzals) have evolved arrays of novel hollow/air-filled (RI 1.0) rod- and pancake-shaped melanosomes from ancestral solid types, leading to an increased refractive index contrast and thereby, extraveriference colors [20]. Some have evolved close-packed 2DPC-like [3] square (peafowl) or hexagonal (ducks and trogons) arrangement of melanosomes [11**][20], although optically they seem to function as multilayers [23, 24]. These diverse melanosomal arrays have convergently evolved numerous times across birds and show complex evolutionary history [11**], with both solid and hollow melanosomes in different plumage patches in some species [21].

Published more than 50 years ago, Durrer and Villiger’s [25**] description of hollow melanosome assembly in barbules of a starling (Lamprotornis) still remains an authoritative source on the ontogeny of iridescent barbule coloration. During feather development, melanosomes produced within specialized melanocytes are dendritically transferred to the barbule plate keratinocytes via endocytosis-like process [20, 25**]. Melanosomes in developing non-iridescent barbules have an exclusion zone around them and seem randomly embedded in a matrix of rapidly polymerizing keratin that physically prevents the migration of melanosomes to the cell boundary [25**]. Whereas, in iridescent barbules, β-keratin is proliferating and polymerizing into small fibrils that do not fuse and remain confined to the center of the cell, with the cytoplasmic melanosomes free to diffuse to the cell membrane. The melanosomes are eventually mechanically confined by the keratin mass as it grows to fill the cell volume, and ordered into a marginal monolayer as the cell flattens upon death and dehydration [25**].

A relatively recent developmental study in Blue-black Grassquit (Volatinia jacarina) observed a greater density of larger, more uniformly-sized melanosomes in barbules of iridescent males relative to non-iridescent females [26]. Based on their observations that the organization of the melanosomes into a flat monolayer occurs late in barbule development, when the cell is dying, they proposed that depletion-attraction forces re-organize melanosomes into a monolayer, as opposed to cellular or molecular mechanisms [26, 27]. When melanosomes aggregate, the volume that keratins are normally excluded from occupies is reduced, increasing entropy and lowering the free-energy of the system (Fig. 2D).

In Lamprotornis sp. [25**], hollow melanosomes are already formed within melanocytes before being transferred to keratinocytes. Premelanosomes, large vesicles (~1 µm diameter) of Golgi origin filled with fine granular tyrosinase are rapidly generated within melanocytes, and later incorporate many small vesicles from the cytoplasm. A zigzag 5-6 layer lamellae forms centrally around which the smaller vesicles are organized like beads on a string. As the premelanosomes flatten, melanin is rapidly synthesized around the foam-like central matrix, which eventually becomes the air-filled internal structure of hollow melanosomes. By contrast, in regular solid melanosomes, melanin synthesis occurs at the zigzag lamellae and completely fills the premelanosome [28]. Another recent study on the ontogeny of hollow melanosomes in iridescent barbules of Wild Turkey (Meleagris gallopavo) indicated that melanosomes are mostly solid and randomly oriented during transport [29]. Once inside the barbule keratinocyte, however, they are mostly oriented in the same direction, and electron-dense material from the core is lost, prior to being close-packed into a hexagonal lattice. Shawkey et al. [29] suggest that in turkeys, melanosomes could be a composite with a phaeomelanin core and eumelanin mantle, and that the chemically unstable phaeomelanin core can degrade upon changes in local environment (e.g., pH). That the mechanisms for hollow melanosome ontogeny are convergent is not surprising given iridescent barbule coloration using hollow melanosomes has evolved numerous independent times across birds [11**][21, 22].

Several outstanding questions remain. Although it is becoming clear that melanosomes late in development assemble via a “crowding mechanism” [27], this needs to be reconciled with spatiotemporal changes in keratin synthesis and polymerization in barbule keratinocytes [25**], which can affect the position of the melanosomes relative to the cell boundary. Furthermore, depletion-attraction alone cannot explain how spaced multi-layers form (Fig. 2E), let alone more complex arrays with double-layered melanosomes in Birds of Paradise [11**][20]. Some authors have argued that the high aspect ratios of melanosomes in iridescent barbules relative to non-iridescent ones is enough for the self-emergence of layering [29, 30]. However, the observed melanosome aspect ratios seem far from the optimum under granular packing considerations of spherocylinders [31]. Moreover, melanosome morphology can vary in a single species more than previously appreciated [11**][20, 21], further confounding these analyses.

Given the key role of melanosomes in iridescent color generation, it is conceivable that convergent regulatory changes in melanin synthesis pathway have led to the re-
peated evolution of barbule iridescence in birds. The genetics of melanin-based coloration is well-studied in animals [32**], and recent progress in butterfly coloration suggest changes to single master regulatory genes can pleiotropically induce structural coloration [7**], while loss of melanin pathway genes can alter the gross morphology of scales themselves [33]. We wonder if similar changes could pleiotropically affect keratin expression and feather morphology in birds. The role of Melanocortin-1 Receptor (MC1R) in determining melanin patterning is inconsistent, but non-coding and coding differences in its repressor, agouti signaling protein (ASIP), is functionally significant across organisms [32**][34]. A recent comparative genomic study on birds of paradise with extravagant iridescent barbule coloration [35] suggests other promising candidates under putative positive selection – ADAMTS20, implicated in melanoctye development through KIT ligand functioning, and ATP7B, involved in copper transport, an element essential for melanogenesis. A similar study in galliforms [36] recovered functional changes in four melanogenesis genes, two of which are KIT and ASIP, but these were not specific to iridescence. More interestingly, they found difference in β-keratin gene expression between white and iridescent green feathers. Recently, melanocytes themselves have been found to autonomously determine color patterning and ASIP expression in adjacent dermal tissue, and this could be investigated via melanocyte transplantation from iridescent to non-iridescent barbule plates [34].

Non-iridescent Feather Barb Coloration

By contrast to iridescent barbule colors (Figs. 1C-D), structural colors in feather bars are usually non-iridescent (Figs. 1E-F) and have evolved in over 45 families across 12 bird orders [11**, 37*]. Two main classes of 3D glassy or quasi-periodic photonic nanostructures are recognized in spongy barb medullary cells (Fig. 2C) – interconnected networks with anastomosing air channels and β-keratin rods (channel-type), and random close-packed arrays of spherical air voids in a β-keratin matrix (sphere-type). Some species with slate to blue-gray plumages have evolved rudimentary barb structures to form is a reticulate matrix of solid keratin fibres with a distinct "crown-of-thorns" appearance, very similar to those seen at the periphery of hollow medullary cells in white feathers, but they are too large to make a visible interference color. Interestingly, the development of non-iridescent feather bars seems identical up until this stage, when cells normally die leaving behind the characteristic foamy-like, pneumatic medulla [42]. In photonic bars, however, development proceeds further and in just over a few hours, the characteristic channel-type network spontaneously appears at the cell boundaries from a cytoplasmic background filled with granular material whose sizes corresponds well with RNPs [42, 43**]. As this polymerizing spongy network grows, the volume occupied by the electron-lucent droplet shrinks. During this process, the barb cells have not yet apopptosed since nuclei remain visible [41**]. When the cells finally die, the cytoplasm, nuclei and other cellular machinery are replaced by air. During this entire process, neither membrane nor cytoskeletal templates or prepatterns were observed directing the assembly of β-keratin into spongy networks, consistent with a phase separation process [41**]. Turing-type patterning, which produces (quasi-)periodic stripes and spots, is another unlikely alternative, as this process usually occurs in 2D (not 3D), and often breaks down with growth over time [39, 44]. Whereas, liquid-liquid phase separation within cells is a growing paradigm to explain the fundamental organization and functioning of cells, including how RNPs can lead to the development of fibrous, self-organized pathologies [43**]. It is plausible that birds have co-opted such innate cellular processes for photonic self-assembly, and future studies will have to investigate the identity and function of RNPs during
FIG. 2. A Schematic for the Assembly of Photonic Nanostructures in Bird Skin (A) and Feathers (B-E). (A) In hierarchical collagen fibrillogenesis [13*] depicted here in increasing levels of spatial organization, diverse triply-helical collagen molecules assemble into composite microfibrils that organize into macrofibrils/fibers, which are synthesized by a single dermal corneocyte [10]. The lower panel shows a dense packing of dermal collagen fibres (blue) oriented parallel to the skin surface with occasional gaps (white), and underlain by a layer of melanosomes. The fibril size, spacing and organization are molecularly determined in vivo, even though collagen can spontaneously self-assemble into fibrils in vitro [13*]. (B) Phase separation of β-keratin is hypothesized to result in spontaneous stereotypical pattern formation (e.g., dendritic spinodal-like patterns depicted) at the periphery of barb cells late in development, when there is a “super-critical” concentration of β-keratin near cell edges due to capillary flow [41**]. (C) Two types of photonic glasses are known in feather barbs - channel- and sphere-type, which are analogous to morphologies seen during spinodal decomposition or nucleation-and-growth [37*]. (D) During iridescent feather barbule development, melanosomes are thought to aggregate, driven by depletion-attraction forces [26, 29]. Note, a small amount keratin molecules trapped in-between the cell membrane and assembling melanosomes could form the thin cortex of iridescent barbules. Timing of keratin synthesis and polymerization within barbule keratinocytes is key to enable the self-assembly of melanosomes [25**]. (E) Melanosomes can be solid or hollow, and can form a thin-film monolayer, densely close-packed or periodically-spaced multilayers. Approximate scale bars are shown alongside biophotonic nanostructures to illustrate the typical spatial periodicities (150-300 nm) needed for light interference.

Feather development and genetic basis of keratin expression are typically studied in Chicken (Gallus gallus), Japanese Quail (Coturnix japonica) and Zebra Finch (Taenopygia guttata) none of which have barb structural coloration, while homology relationships between and among α- and β-keratins are only just being uncovered [45, 46*]. Across birds, there is extreme variation in copy numbers of β-keratin genes (6 in owls - 149 in Zebra Finch; average 34). A complex pattern of differential expression of different types of keratin genes (scale and claw, feather, keratinocyte) from multiple chromosomal loci in different feather tissues has been documented [45, 46*], with many of the feather β-keratin genes evolving their own chromosome-wise transcription factors [47]. Nevertheless, barb ridge specific (e.g., barb vs. barbule) expression profiles are unavailable [46*]. We believe the key to unlocking the molecular basis for β-keratin self-assembly lies in generating tissue-specific, time-resolved expression of feather and feather-associated keratins, and identifying copy number variation unique to each family/genus [36][46*][48]. Different family-specific combinations of β-keratins might confer different macromolecular properties (e.g., hydropathy, charge) that can aid or...
hinder self-assembly, while tuning the stoichiometry of expressed keratins may predictably determine the length scale at which the phase separation arrests, which is crucial for color production. Comparatively studying the tissue-specific molecular structure of feather keratins or looking for differences in the macro-molecular packing of β-keratin filaments, for instance, in spongy photonic barbs vs. hollow white barbs or barbules could also help illuminate the structure-function relationships underpinning keratin self-assembly.

CONCLUSIONS AND FUTURE DIRECTIONS

Major aspects of organismal structural color pathways remain currently opaque. Nevertheless, we have highlighted how birds appear to have co-opted developmental programs behind collagen fibrillogenesis in dermis, melanosome synthesis and inclusion in feather barbules, and keratin polymerization in feathers barbs, to produce structural coloration. The redundant regulatory control of fibrillogenesis, melanosynthesis and cornification provides alternative pathways that can be modified by selection. This could explain the repeated convergence of avian structural coloration. We have also discussed the important role played by short-ranged attractive and long-ranged repulsive forces typically seen in soft colloidal systems [9][27][43**][49], in feather nanostructure development.

Interrogating molecular regulation of collagen fibril spacing by manipulating collagen fibrillogenesis [13*] is one future challenge that is tractable in Silky Chicken (artificially-selected variant of model Gallus gallus), with its hypermelanized dermis and unique blue earlobes. Another direction is to determine the exact role played by high melanosome densities in self-assembly during iridescent barbule development [26]. The putative function(s) of cytoplasmic RNPs in flocculation and/or self-arrest also needs to be investigated [43**][49]. Comparative transcriptomics and genome-wide association studies might represent promising complementary approaches to cell and developmental biology. Two of ~50 bird species with published complete genomes have barb structural coloration, while at least a quarter have barbule iridescence [50]. As successfully demonstrated for pigmentary coloration [36], these methods could help identify candidate genes, whose functions can be tested using latest genome-editing technologies (e.g., CRISPR-Cas9) in existing model species with iridescence – Silky, Domestic Chicken, and Turkey.

A burgeoning number of studies in physics and engineering are looking to organismal structural coloration, which have been evolutionarily optimized over millions of years of selection, as a rich reservoir for the bioinspired design and synthesis of functional materials, given current challenges in sustainable manufacture and synthetic self-assembly at visible optical length-scales [9][51]. Genetic and developmental knowledge of biophotonic nanostructures may lead to next-generation technologies that directly biomimic in vivo self-assembly using bio-similar and biodegradable materials in vitro.

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Declaration of Interests: The authors declare no conflict of interests.

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**BOX 1: GLOSSARY**

**Refractive Index** – this dimensionless metric describes the amount of retardation of light in a dense medium relative to vacuum. The RI determines the extent to which light rays are bent (refraction) when entering a dense material from air. See [3].

**Biophotonic Nanostructure** – a nanoscale feature in the animal integument with random, quasi-periodic or periodic variation in material composition that leads to a refractive index contrast (e.g., β-keratin and air). See [1][11**].

**Photonic Crystal** – A concept borrowed from solid-state physics, a photonic crystal describes periodic variations in material composition that interferes with the propagation of light through the material. See [1, 3].

**1D, 2D, 3D** (photonic materials) – The designation 1D, 2D or 3D describes whether the material varies in composition or refractive index along one (e.g., a thin-film or multilayer lamellae), two (e.g., square or honeycomb lattices of cylindrical holes), or three (e.g., gem opals and cubic crystals) principal orthogonal directions (e.g., Cartesian axes). For instance, in a 2D columnar or fibrillar nanostructure, there is no variation along column/fibril axis (say z-axis), but there is in the cross-sectional plane (along x and y). See [1, 3][11**].

**Iridescence** – a change in hue with angle of illumination or angle of observation.

**Self-assembly** – a ubiquitous phenomenon that describes the spontaneous or emergent organization of materials at macro-molecular length scales, usually driven by thermal fluctuations and interactions among molecules.

**Phase separation** – a process that describes the spontaneous unmixing of immiscible binary or ternary mixtures, under unfavorable conditions. See [43**].

**Depletion-atraction** – a process that describes the attractive force that brings together larger colloidal particles that reduces the excluded volume around the large particles that a competing smaller solute (depletant) cannot normally occupy, thereby increasing the positional entropy for the depletants and lowering the overall free-energy of the system. Depletion-atraction can also lead to phase separation. See [49].