CHAPTER THREE

Advanced Methods for Studying Pigments and Coloration Using Avian Specimens*,†

Kevin J. Burns, Kevin J. McGraw, Allison J. Shultz, Mary C. Stoddard, and Daniel B. Thomas

Abstract. Advanced analyses of feathers, eggs, and other colorful tissues in ornithology collections are revealing fresh insights into the life histories of birds. Here, we describe the methods used in these studies, including high-performance liquid chromatography, digital photography, Raman spectroscopy, and spectrophotometry. We use case studies from across the diversity of birds and from deep in the fossil record to illustrate method usage, limitations, and other considerations for analyzing museum specimens. Structural colors in feathers and the surface coloration of eggs are particularly emphasized.

Key Words: digital photography, egg pigmentation, high-performance liquid chromatography, hyperspectral imaging, Raman spectroscopy, spectrophotometry, structural coloration.

Colors are essential for the life history strategies of many animals, including birds (Hill and McGraw 2006a,b). Bright and vivid colors often function as visual signals, communicating the quality of an individual to potential mates, rivals, or predators (Hill 1991, Pryke and Griffith 2006, Maan and Cummings 2012). Muted and dark colors also can be important social signals (Møller 1987, Hoi and Griggio 2008, Karubian et al. 2011), or provide camouflage to animals at both lower and higher trophic levels (Götmark 1987, Montgomerie et al. 2001, Charter et al. 2014). These and other fitness benefits have driven the evolution of a remarkable diversity of pigments and color-producing structures among animals (McGraw 2006a,b; Prum 2006). For their intricate mechanisms of color generation, and the do-or-die importance of their colorful displays, birds are particular marvels of coloration. Birds have colorful eggs, eyes, plumage, skin, and scales, and can show substantial variation in coloration across species, populations, and individuals.

The significance and evolution of color variation among birds is often revealed through comparative analyses, and ornithology collections are ideal for large-scale comparisons of color and other phenotypes. The new layers of information added to ornithology specimens through analyses of plumage and eggshell color exemplify the "extended specimen" philosophy. Spectrophotometry, digital photography, chromatography, and Raman spectroscopy provide

* Burns, K. J., K. J. McGraw, A. J. Shultz, M. C. Stoddard, and D. B. Thomas. 2017. Advanced methods for studying pigments and coloration using avian specimens. Pp. 23–55 in M. S. Webster (editor), The Extended Specimen: Emerging Frontiers in Collections-based Ornithological Research. Studies in Avian Biology (no. 50), CRC Press, Boca Raton, FL.

† All authors contributed equally to the chapter.

detailed information about the light absorbance properties and pigment compositions of tissues. Accordingly, the development of these sophisticated new methodologies is opening new doors for the use of museum specimens in the analysis of pigmentation and coloration. And yet each technique also has characteristic advantages and disadvantages when studying the colors of feathers, eggshells, or other tissues.

In this chapter, we describe the modern toolkit for studying avian coloration from museum specimens. We begin with spectrophotometry, a fundamental technique for analyzing colorful tissues. From underlying principles to data analysis, we describe the use of spectrophotometry in ornithology collections and identify future areas of research. Spectrophotometry is showcased in special case studies on structural coloration and avian eggshell coloration. Next we discuss advances in digital photography and hyperspectral imaging, which are quickly becoming critical tools for the study of animal coloration. We next transition from surface coloration to the underlying chemistry of coloration: the chromatography section summarizes recent advances in analysis of avian pigmentation. High-performance liquid chromatography is highlighted as the gold standard technique for identifying carotenoids, porphyrins, and other pigments in avian tissues. The next section of the chapter focuses on Raman spectroscopy, a relatively unknown technique in ornithology. The key advantage of laser-based Raman spectroscopy is nondestructive analysis, which can be ideal for precious museum specimens. The final section of the chapter highlights the emergence of new methods, including computer vision algorithms to analyze egg patterns, for the study of eggshell coloration. Such methods can extend to the study of plumage. Collectively, these sections give an overview of the advanced techniques currently used to study avian coloration from museum specimens as well as living animals.

SPECTROPHOTOMETRY

The study of plumage coloration has a long history in ecology and evolutionary biology; until recently most studies have relied on human perception of avian color. Given the subjective nature of human assessments, researchers began arguing that the quantification of reflectance spectra provided a superior measure of avian color than is possible with human observers (e.g., Endler 1990, Johnson et al. 1998). The use of spectrophotometry has become even more important with the acknowledgment that human vision and avian vision differ in two major ways. First, unlike humans who only have three types of retinal cones, birds have four types with different spectral sensitivities (Vorobyev et al. 1998, Cuthill 2006). One of the avian cone types is most active in the ultraviolet (UV) portion of the spectrum, enabling birds to see into a portion of the spectrum that humans cannot perceive (Goldsmith 1980, Cuthill 2006). Second, birds also have oil droplets attached to each of these cone cells that act as long-pass cut-off filters that absorb all wavelengths of light below certain values, thus enhancing birds' discriminatory capabilities (Vorobyev and Osorio 1998, Vorobyev et al. 1998, Vorobyev 2003, Hart and Vorobyev 2005, Cuthill 2006).

Although scientists have appreciated these differences between birds and humans for many years, only recently have studies of plumage coloration incorporated this information through reflectance spectrophotometry (Cuthill 2006, Bennett and Théry 2007). Reflectance spectrophotometry studies have shown that quantification by human vision alone does not provide sufficient information for the study of avian plumage coloration (Bennett et al. 1994, Cuthill et al. 1999, Cuthill 2006, Håstad and Odeen 2008). For example, sexual dichromatism might be much more prevalent than can be appreciated by human perception (Andersson et al. 1998, Cuthill et al. 1999, Eaton 2005, Eaton and Johnson 2007, Burns and Shultz 2012). Humans might be able to accurately measure dichromatism, but only with some types of coloration (i.e., not UV), and it is difficult to predict under what conditions humans can discriminate dichromatic birds (Armenta et al. 2008b, Seddon et al. 2010). Similarly, several studies (e.g., Shultz and Burns 2013) have reported correlations between UV plumage signals and factors such as mating systems and habitat. Thus, studies that rely on human vision alone to investigate potential correlates to avian coloration are ignoring a potentially critical component of the signaling system of birds. Likewise, spectrophotometry provides a more objective and detailed approach to studies of geographic variation and taxonomy than human vision can provide (e.g., Schmitz-Ornés 2006).

Museum collections facilitate these studies by providing the large series of specimens needed—both within and across species—for detailed comparisons. It is often not practical nor possible for one researcher to collect such a series during a reasonable time span. For example, using museum specimens, Eaton (2005) assessed a broad array of birds to provide a general evaluation of the prevalence of sexual dichromatism across all birds, and Burns and Shultz (2012) surveyed nearly all species in one large group (Thraupidae, 372 species) to provide an assessment of the prevalence of UV plumage patches.

Limitations and Considerations for Using Specimens

Museum specimens can be a wonderful resource for filling in sampling gaps or surveying a wide range of species, but special considerations must be kept in mind when measuring coloration. Many studies of bird coloration focus on plumage coloration, but coloration in other body parts, such as bare skin, the bill, legs, or irises, may also play an important role in signaling and behavior (e.g., Murphy et al. 2009). Unfortunately, colors in these soft tissue parts are rarely preserved in museum skin specimens, as the integument dries out and eyes are rarely preserved. Plumage coloration is generally well preserved if specimens are stored in appropriate conditions including protection from insects, light, and damage from other abiotic factors. But even if stored in appropriate conditions, fading and color changes can still occur, particularly in older specimens (Hausmann et al. 2003, McNett and Marchetti 2005, Armenta et al. 2008b, Doucet and Hill 2009). Disagreement exists about the extent of change in coloration under differing storage conditions. For example, Hausmann et al. (2003) found no particular change in the UV part of the spectrum as specimens aged, whereas McNett and Marchetti (2005) found greater degradation of reflectance spectra at shorter wavelengths in older museum specimens. Dissimilarities in the types of changes can be observed in regions colored by different mechanisms (Doucet and Hill 2009). Potential storage and age-related effects are primarily relevant for intraspecific studies, as changes are typically small (Doucet and Hill 2009). By choosing specimens from similar time periods and museums, effects from changes in coloration due to storage can be minimized. Finally, coloration changes are less severe in younger specimens, and Armenta et al. (2008a) demonstrated that specimens younger than 50 years old have spectral feather measurements similar to those of live birds.

The approach used to capture spectrophotometer measurements has remained largely unchanged for the last decade, and a detailed description of the equipment and setup is reviewed by Andersson and Prager (2006). However, note that iridescent colors, or colors with a hue that is angle-dependent, can be difficult to measure in an accurate and repeatable manner (Meadows et al. 2011). Thus, the conclusions of a study may depend on angle geometry if the angle of reflectance is not properly taken into account (Santos and Lumeij 2007). Nonetheless, by controlling for angle and quantifying maximum reflectance, it is possible to obtain highly repeatable measurements within an individual specimen, even for iridescent colors (Meadows et al. 2011).

Analytical Approaches

After obtaining raw reflectance spectra, a number of different ways exist to analyze the data and extract variables that can be used in studies of evolution, ecology, and behavior. Historically, a popular way to describe colors has been to calculate tristimulus color variables such as hue, saturation, and brightness (Montgomerie 2006), which are extracted directly from the reflectance spectrum. For example, "hue" is calculated as the wavelength of highest reflectance. In terms of color perception, hue depends on the relative stimulation of color cones, so the tristimulus "hue" value may not relate well to the "hue" actually perceived by the receiver, particularly when a reflectance curve has more than one peak (Montgomerie 2006). Depending on which tristimulus metrics are used, it can be very difficult to compare values across patches, much less across studies (Montgomerie 2006, Delhey et al. 2014). Principal component analysis (PCA) can also be used to describe variation across raw reflectance spectra (Montgomerie 2006) without any sensory system assumptions. Although PCA can be a useful way to objectively identify intra- or interspecific variation, it also has several drawbacks, including variation in brightness swamping out other signals, the inability to analyze spectra from different colors due to difficulty interpreting subsequent

PC axes, and lack of comparability across studies of other species or even other patches of the same species (Montgomerie 2006). The benefit to using methods based on raw spectral curves, such as tristimulus metrics or PCA, is that they make no assumptions about a particular visual system. This approach can be useful for studies of color production (e.g., Shawkey and Hill 2005) and systematics (e.g., McKay 2013), and can also be useful when little is known about the visual system of the intended signal receiver(s).

An alternative is to quantify reflectance spectra while incorporating details about the sensory perception of the receiver (Montgomerie 2006). Today it is common for researchers to incorporate models of avian perception when analyzing color. A number of approaches exist. We will discuss the two most common approaches later: chromaticity diagrams or tetrachromatic color space models and receptor-noise discrimination models. Both methods hinge on the calculation of the quantum or photon cone "catch," or total output, of each of the four color receptors (Cuthill 2006, Montgomerie 2006), which requires information about the spectral sensitivities of the four avian color cone types. These models can also include detailed information about oil droplets in the avian color cones, the irradiance spectrum of incident light, and the transmission properties of air and the bird's ocular media (Montgomerie 2006).

Often the spectral sensitivities of the species in question are not known, so researchers may use the most closely related species with this information, such as the Blue Tit (*Cyanistes caeruleus*) in the case of passerines (Hart et al. 2000). Spectral cone sensitivities are thought to be highly conserved, though there are two broad categories for the cone sensitive to the shortest wavelengths (Hart 2001). This cone can either be most sensitive in the UV wavelength range (UVS visual system) or shifted upward toward the violet wavelength range (VS visual system) (Cuthill 2006). Recent work sequencing the SWS1 opsin gene that includes the single nucleotide polymorphism correlated with this sensitivity shift has shown that the UVS/VS visual system has shifted across families in the bird phylogeny multiple times (Ödeen and Håstad 2013) and that the visual system can shift even within a relatively small family of birds (Maluridae; Ödeen et al. 2012). However, few species' visual systems have been physiologically or behaviorally characterized to quantify visual system variation (Kemp et al. 2015). Note that, in addition to the four single-cone types described earlier, birds also have double cones that are thought to be important in the detection of achromatic (luminance) signals, such as motion perception and the detection of pattern, texture, and form (Osorio and Vorobyev 2005, Hart and Hunt 2007). Because chromatic and achromatic cues are likely processed independently in birds (Vorobyev and Osorio 1998, Kelber et al. 2003, Endler and Mielke 2005), often luminance achromatic signals are modeled separately when considering signal contrasts (e.g., Doucet et al. 2007). Once calculated, the quantum cone catches are generally applied in one of two ways: they can be analyzed in avian tetrahedral color space (Goldsmith 1990, Endler and Mielke 2005, Montgomerie 2006, Stoddard and Prum 2008, Kemp et al. 2015, Renoult et al. 2015) or used in calculating discrimination thresholds, such as just noticeable differences (JNDs; Vorobyev and Osorio 1998). These different approaches are reviewed in Kemp et al. (2015) and are described in more detail later.

The avian tetrahedral color space (Endler and Mielke 2005, Endler et al. 2005, Stoddard and Prum 2008) is a kind of chromaticity diagram. For a given color patch, the quantum cone catches are calculated and transformed in a tetrahedron whose vertices represent each of the four photoreceptor classes (Figure 3.1), thus indicating the extent to which each cone class is stimulated. Within the avian tetrahedral color space, the vector drawn from the achromatic center to the color patch measurement can then be described with spherical coordinates. These coordinates include theta (or longitudinal measure) and phi (or latitudinal measure), both proxies for hue; and r (the length of the vector), a proxy for chroma (saturation; Stoddard and Prum 2008). In addition, by plotting all plumage regions from an individual or species together, it is possible to obtain measurements to describe the entire occupied color space using a series of measurements (Figure 3.1). These measures include color span, which is the distance between patches; hue disparity, which is the difference in vector angles; and color volume, which is the volume of the minimum convex polygon occupied by all plumage measurements (Figure 3.1; Stoddard and Prum 2008). One can also calculate the overlap of different

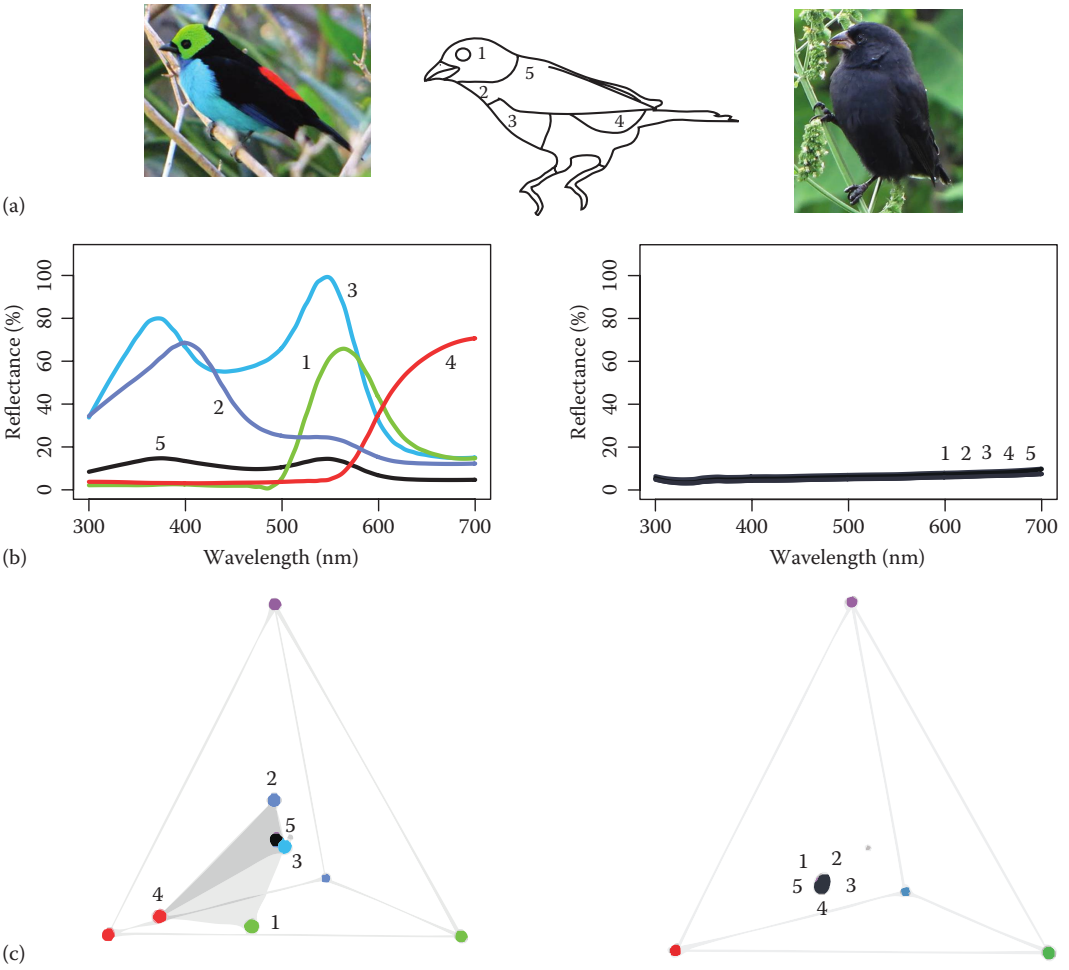

Figure 3.1. Spectrophotometer measurements from the Paradise Tanager (*Tangara chilensis*; left column) and the Small Ground Finch (*Geospiza fuliginosa*; right column). Measurements were taken from the crown (1), throat (2), breast (3), rump (4), and back (5), as depicted on the line drawing (top center). (a) Photographs of the birds show that, from a human perspective, the Paradise Tanager is quite colorful with many different color patches, whereas the Small Ground Finch is quite drab with uniform black plumage. (b) Spectrophotometer measurements show that the reflectance curves from the Paradise Tanager vary across body regions, and are likely produced by different coloration mechanisms that include feather structure (2 and 4), carotenoids (4), melanin (5), and a combination of structure and carotenoids (1). In contrast, the Small Ground Finch, consistent with the human view, has very similar reflectance curves from all regions and are all likely produced by melanin. (c) When plotted in the avian tetrahedral color space (plotted using pavo; Maia et al. 2013a), the Paradise Tanager plumage regions occupy a much greater proportion of the color space and therefore have a much greater color volume (depicted by the gray polygon), color span, and hue disparity. For the Small Ground Finch coloration, on the other hand, the plumage regions all cluster together in the avian tetrahedral color space, and have a small color volume, color span, and hue disparity. (Photos by K. J. Burns.)

color distributions (Stoddard and Stevens 2011) and quantify the full breadth of signaling in taxonomic groups (Stoddard and Prum 2011, Shultz and Burns 2013; see upcoming case study). Ultimately, tetrahedral color space analyses are powerful because they provide a way to make first approximations of color differences, make fewer assumptions than discrimination models (described in the next section), and provide a convenient way to visualize color variation across individuals, species, and broader taxonomic groups.

For determining whether two color stimuli can be discriminated, a second type of visual

model is used: a receptor-noise limited model that models color differences in terms of JNDs (Vorobyev and Osorio 1998). The model is based on the idea that color discrimination is limited by noise arising in the photoreceptors, and early experiments showed that behavioral performance could be predicted, under some conditions, based on estimates of noise in each channel (Vorobyev and Osorio 1998, Vorobyev et al. 2001). The model produces a measure of the difference between two color stimuli, measured in JND, where a JND value less than one means that the colors are indistinguishable and a JND greater than one means that the colors can be discriminated. The model is very useful for asking questions about fine-scale color discrimination, which is highly relevant for questions about mimicry, crypsis, and mate choice. The Vorobyev-Osorio JND model is designed to describe color differences at or near threshold (i.e., two very similar colors); however, it is not known how well the model explains suprathreshold color variation. The question of how best to model colors that are quite different remains an open one (Kemp et al. 2015), given that we still know relatively little about neural color processing (Renoult et al. 2015) such as color constancy (Kelber and Osorio 2010). For estimating suprathreshold color differences in the absence of explicit behavioral data, JND measurements are unlikely to provide advantages relative to measurements derived from chromaticity diagrams (Kemp et al. 2015).

The R package, pavo, is useful for performing many of the analyses described in the preceding sections, and can be easily incorporated into scripts to automate analyses across many species (Maia et al. 2013a). The TetraColorSpace program (Stoddard and Prum 2008) in MATLAB® also provides users with a menu of options for analyses in tetrahedral color space.

After measurements are collected from raw reflectance data (tristimulus color variables, quantum cone catches, or avian tetrahedral color space measurements), PCA can be used on these calculated values to identify which components of coloration explain the most variation among individuals (Montgomerie 2006, Delhey et al. 2014) or among species (Mason et al. 2014). These principal component values can also be used in downstream analyses to simplify interpretation and to perform fewer tests on individual variables to minimize the risk of false positive results (e.g., Mason et al. 2014).

Applications to Ecology and Evolution

Spectrophotometry has been applied to address a variety of questions of ecological and evolutionary importance. These include studies of sexual selection (e.g., Eaton and Johnson 2007, Mason et al. 2014), species delimitation (e.g., Schmitz-Ornés 2006, Maley and Winker 2007), seasonal changes in color (e.g., Tubaro et al. 2005, Barreira et al. 2007, Delhey et al. 2010), age-related social status (e.g., Bridge et al. 2007, Nicolaus et al. 2007), and to assess the relationship between plumage and ecological characteristics (e.g., Friedman et al. 2009, Shultz and Burns 2013, Dunn et al. 2015). Here we highlight a case study to illustrate the types of questions that can be addressed with spectrophotometry of museum specimens.

Plumage color may be shaped over evolutionary time by the particular light environment found in the habitat of a species. For example, selection may favor crypsis, with plumage color evolving to match that of the background (Endler and Théry 1996, Doucet et al. 2007), whereas conspicuousness might be favored in other situations (Marchetti 1993, Gomez and Théry 2007). Species can also experience a variety of light environments within broad habitat characterizations. For example, species found in the same forest habitat that spend more time in the canopy are subject to a different light environment than those in the understory (Gomez and Théry 2007). By objectively quantifying plumage color, spectrophotometry facilitates the study of these diverse selection pressures across species.

Shultz and Burns (2013) addressed the effect of the light environment on plumage color evolution in a group of 44 species of tanagers in the subfamily Poospizinae. They quantified plumage in these species from museum skins, and then mapped aspects of plumage color across a molecular phylogeny. They compared different models of trait evolution with varying degrees of complexity, including models where habitat had no effect, models that compared open versus closed habitats, and models that incorporated foraging strata as well as open versus closed habitats. Plumage was quantified by plotting reflectance spectra in the avian tetrahedral color space (Stoddard and Prum 2008) and extracting values

for mean color span, color volume, mean hue disparity, mean chroma (saturation), and mean brilliance (brightness) for each sex of each species. The model-fitting results showed that habitat plays an important role in shaping the plumage of both males and females. Plumage measures of color diversity best fit a model that only included the selective regime of open versus closed habitat, but measures of plumage brightness best fit a model that included foraging strata as well as open versus closed habitat. These results suggest that species within this clade match background contrast and color diversity to increase crypsis, and that the way that this is achieved depends on environmental lighting variables.

Future Directions

As more spectral data are accumulated, we will need more complex methods and models to analyze these types of data in a meaningful way, so that they can be incorporated more broadly into studies of ecology and evolution. When collecting spectral data, it will be essential to archive and annotate the raw data in a manner that will be accessible to researchers in the future (e.g., the Dryad database; White et al. 2015). Finally, while reflectance spectra currently represent the most accurate way to quantify plumage coloration in many circumstances, digital photography (see following section) is becoming more popular and offers several advantages over spectrophotometry, such as the ability to consider not only color but also patterning and patch sizes (Stevens et al. 2007, McKay 2013).

DIGITAL PHOTOGRAPHY AND HYPERSPECTRAL IMAGING

Digital photography is quickly becoming a powerful tool for visual ecologists, due to the ease and speed with which it allows two-dimensional information to be captured and analyzed (Stevens et al. 2007, Pike 2011, Akkaynak et al. 2014). Unlike spectrophotometry, which allows for point-by-point color capture, digital images simultaneously capture color and spatial information. Once photographs are obtained, digital image analysis can be performed, often using custom-designed computer code for analyzing traits of interest. Digital photography has been employed in several recent museum-based studies involving egg coloration and patterning (Cassey et al. 2010a, 2012a; Stoddard and Stevens 2010; Stoddard et al. 2014; also see the upcoming section "Advanced Methods for Studying Avian Egg Color"). Researchers have also used digital photography in skin collections to investigate the extent to which avian taxa differ in plumage coloration (McKay 2013, McKay et al. 2014), demonstrating the great potential of digital photography as a tool for systematics. Additionally, digital photography has proven useful for the quantification of more complex aspects of plumage appearance, including barring (Gluckman and Cardoso 2009).

To make a camera ready for use in studies of animal coloration, a series of custom calibrations and corrections must be performed (Stevens et al. 2007, 2009). In particular, calibrations typically involve linearizing and equalizing the RGB responses for each channel and determining the specific sensitivities of the camera's different sensors. To study avian colors using digital photography—as in spectrophotometry—it is important to capture light across the entire bird-visible range of wavelengths (approximately 300 to 700 nm). Note that many other animal taxa, including many reptiles, amphibians, fish, and insects, also have ultraviolet sensitivity. Capturing the full visible spectrum using a digital camera is usually achieved by taking one image with a visible pass filter to block the UV and infrared, and a second image through a UV-pass filter, which blocks non-UV wavelengths; these two images can then be combined to cover the full range of wavelengths required. Most cameras contain a UV-blocking filter, which must be removed, and care must be taken to use a lens that can transmit ultraviolet wavelengths. The main drawback to digital photography is that the modification and calibration process can be complex and time-consuming. However, once these steps are completed, a camera can be used to efficiently gather color data, including ultraviolet, in the field and in museum collections, and these data can then be analyzed objectively or with visual models.

New software packages designed for color analysis make digital photography increasingly attractive (Troscianko and Stevens 2015) for color studies. In addition, tools for the analysis of pattern and texture in digital images are becoming increasingly popular. For example, Stoddard et al. (2014) recently developed a pattern recognition and matching tool called NaturePatternMatch

(www.naturepatternmatch.org) for the analysis of complex visual signals. The tool uses the scale-invariant feature transform (SIFT; Lowe 1999, 2000), a computer vision algorithm designed to detect informative local features in an image. These features are extracted and then matched across images. NaturePatternMatch is inspired by visual processes believed to be important in vertebrate recognition tasks, though more work needs to be done to establish which model of computer vision most accurately resembles true visual recognition in birds and other animals. Ultimately, NaturePatternMatch can be used to understand aspects of animal signaling, recognition, and camouflage, as well as to explore aspects of avian pattern formation and development.

As museums move to digitize their skin and egg collections, curators are advised to consider using carefully calibrated cameras. At the very least, color charts (such as those made by X-rite, Grand Rapids, MI) should be included as color standards in digital photographs of specimens. Note that although digital photography with proper calibrations can permit objective color measurements and, if combined with visual models, estimates of avian retinal cone stimulation values, it is not possible to reproduce the full reflectance spectrum of a given color, as is the case with a spectrophotometer. In this sense, spectrophotometry and digital photography both have an important and complementary place in the study of avian coloration. To achieve full spectral capture in two or three dimensions, a hyperspectral camera is required.

Hyperspectral cameras, which capture full spectrum information at each pixel in an image, have been developed (Chiao et al. 2011, Kim et al. 2012) and may soon become the gold standard for quantifying avian coloration. Already, hyperspectral imaging has been incorporated into field-based studies of animal coloration and camouflage (Chiao et al. 2011, Russell and Dierssen 2015). However, hyperspectral cameras are expensive and require sophisticated postcapture processing. In the future, it will be critical to develop advanced computational methods for analyzing hyperspectral data in a meaningful and efficient way.

As a final and important point, we strongly urge researchers not to rely on color plates, illustrations, or uncalibrated photographs when addressing questions about avian coloration in the context of signaling and communication. Not only do these media fail to convey information about ultraviolet coloration, they are highly variable, subjective, and sometimes misleading. Carefully controlled spectrophotometry, digital photography, and hyperspectral imaging—applied to specimens in the field or in museum collections—are critical for the correct and rigorous assessment of avian color signals.

CHROMATOGRAPHIC ANALYSES OF BIRD PIGMENTS

Some of the most striking colors displayed by birds are derived from chemical pigments deposited in integumentary tissue. Though we now know that many structural features of avian tissues also can play an integral role in color production (see later), much of the early work on the mechanisms of avian coloration centered on the types of pigments—some of which are endogenously produced and others of which are environmentally acquired—used to generate the array of colors seen in bird feathers and bare parts, including the beak, iris, eye ring, and legs. At least six major classes of avian integumentary colorants exist—carotenoids, melanins, psittacofulvins, porphyrins, pterins, and turacins (Figure 3.2)—and, due to their unique molecular characteristics, a host of biochemical procedures are available to analyze the colorants of birds. Recent technological improvements, including high-performance liquid chromatography (Stradi et al. 1995a) and Raman spectroscopy (see later; Stradi et al. 1995b), have aided in both identifying previously undiscovered compounds and in quantifying amounts of both major and minor forms of these pigment types, so that refined questions can be asked about the control, function, and evolution of avian pigmentation. Museum specimens have served as rich storage depots of material, especially feathers and eggshells (Thomas et al. 2014b, 2015), for extracting and analyzing pigments, and for testing hypotheses about the evolution of pigment-based color mechanisms and how this links, for example, to variation in coloration, ecology, phylogeny, and sexual dichromatism.

Analytical Approaches

Avian integumentary pigments were among the first pigments described in animals, just a few

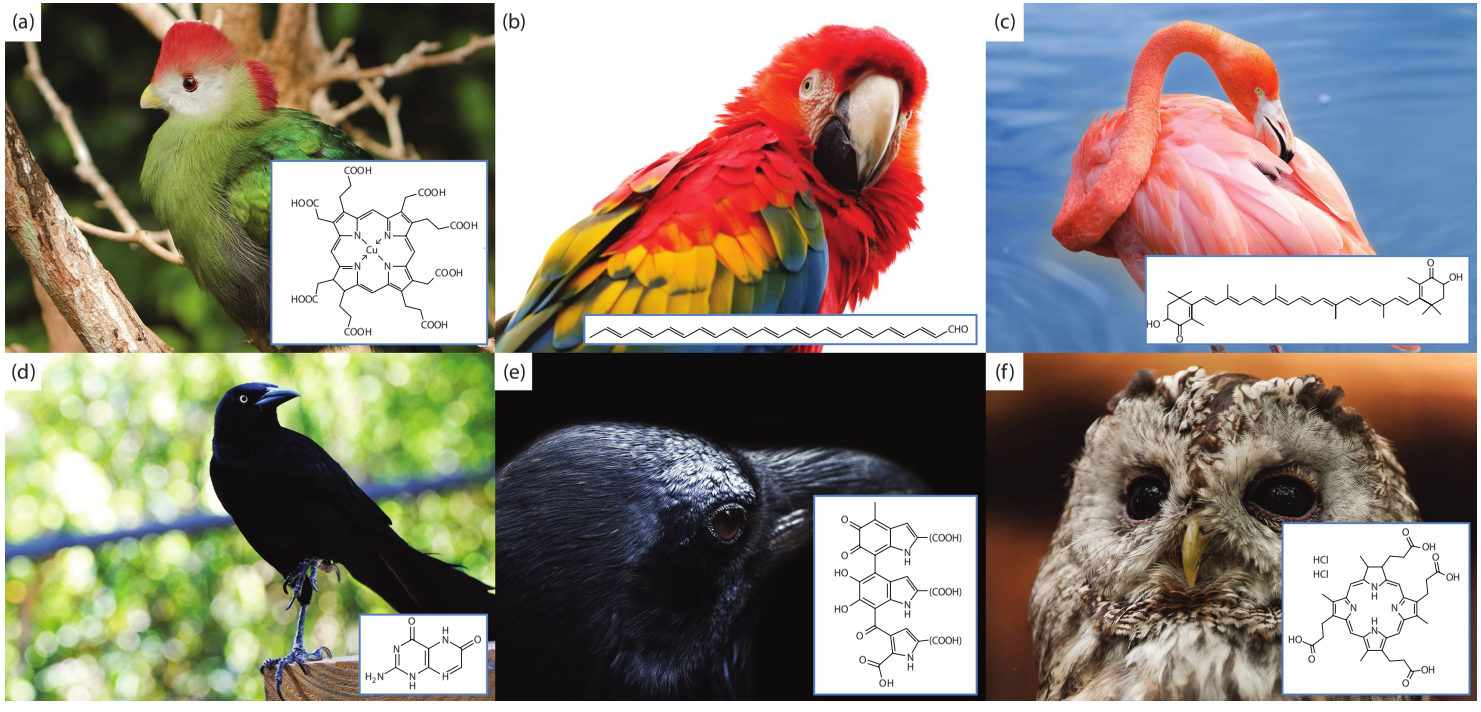

Figure 3.2. Diversity of pigments found in avian integument. (a) Red turacin (molecular structure depicted) and green turacoverdin in the plumage of a Red-crested Turaco (*Tauraco erythrolophus*); (b) red psittacofulvins in the plumage of Scarlet Macaws (*Ara macao*); (c) carotenoid pigment (molecular structure of astaxanthin depicted) in the pink plumage of American Flamingos (*Phoenicopterus ruber*); (d) pterin pigment (molecular structure of xanthopterin depicted) in the yellow iris of a male Great-tailed Grackle (*Quiscalus mexicanus*); (e) melanin pigment (incomplete molecular structure of eumelanin depicted; arrow at top denotes diverse functional groups that can be included) in the black feathers of Common Raven (*Corvus corax*); and (f) porphyrin pigment (molecular structure of coproporphyrin III depicted) in the brown plumage of a Tawny Owl (*Strix aluco*).

decades after carotenoids were described for carrots (Wackenroder 1831). The first biochemical investigations of avian pigments were done over a century ago on the unusual colorants in feathers of turacos (Musophagidae; Church 1869, Gamgee 1895) and parrots (Psittaciformes; Krukenberg 1882; Figure 3.2). These analyses, which predate chromatography techniques (Tswett 1906), were largely restricted to simple chemical testing, such as acid-base-heat reactivity and phosphate precipitation, and general spectral characterization, rather than molecular characterization *per se*. Interestingly, turacos and parrots have been found to be the only groups of animals that harbor these particular pigment classes (turacins and psittacofulvins, respectively); these rare, autapomorphic expressions of pigmentation merit further investigation, especially with respect to their distribution within each taxonomic group (sensu McGraw and Nogare 2005) as well as their molecular and genetic underpinnings.

Once adsorption chromatography became more accessible to chemists in the 20th century (e.g., Strain 1934), it facilitated the identification of particular types of pigments in birds. Different forms of carotenoids, for example, were separated from the plumages of bird groups ranging from woodpeckers (Völker 1934, Test 1969) to bishops (Kritzler 1943), and it was at this time that diet experiments coupled with feather analyses showed that birds could deposit both dietary forms, such as lutein and zeaxanthin, and metabolites, such as picofulvin, into colorful plumage. Adsorption chromatography also permitted the first elucidation of unique fluorescent porphyrins in the rufous-colored plumage of some birds, such as owls and bustards (Figure 3.2; Völker 1938).

Thin-layer chromatography served as the popular method for analyzing bird pigments throughout much of the mid-20th century, with work on carotenoids again grabbing the majority of attention by avian pigment chemists. Fox, Volker, and Brush were instrumental in using this technique to characterize additional feather carotenoids from new avian taxa (e.g., Ciconiiformes, Fox 1962; Cotingidae, Brush 1969) and in enabling biological inquiries such as the pigmentary origin of sexual dichromatism (Brush 1967), interspecific differences in coloration (Troy and Brush 1983, Hudon et al. 1989), intraspecific genetic plumage variation (Brush and Seifried 1968, Brush 1970), and the specific precursor–product relationships for plumage carotenoids modified from dietary forms (Fox et al. 1969, Brush and Power 1976). Other than egg yolk and skin of chickens (Smith and Perdue 1966), and a few species of game birds (Czeczuga 1979) and wading birds (Fox 1962), soft tissue pigments of birds had been largely ignored. A major finding from this initial work, though, was that the fatty-acid esters of carotenoids, such as astaxanthin in pheasants, can be found in avian skin, indicating that birds may need to stabilize pigments in these bioactive tissues if they are to display them. This era also brought refined chemical analyses of feather porphyrins in owls and bustards, such as the identification of free and esterified forms of coproporphyrin, uroporphyrin, and protoporphyrin (With 1978); and the identification of new compounds including pterins and purines that create white, yellow, orange, and red color in the avian iris (e.g., Oliphant 1987), though hemoglobin and carotenoids can, instead, create these colors in some species (Oliphant 1988).

The invention of high-performance liquid chromatography (HPLC), coupled with improved extraction techniques (Hudon and Brush 1992) and the availability of pure standards isolated from organisms or synthesized chemically, opened the floodgates for easier, less expensive, and more extensive separation of avian integumentary pigments starting in the early 1990s (Hudon 1991, Hamilton 1992) and continuing today (e.g., Prum et al. 2014). Again, the vast majority of research applying HPLC centered on carotenoids. Stradi and colleagues pioneered the use of HPLC in their extensive descriptions of carotenoids in the cardueline finches (Stradi et al. 1995a,b, 1996, 1997) and woodpeckers (Stradi et al. 1998). This foundation of work, coupled with the growing understanding of the diverse biological roles that carotenoids can play in animals (Lozano 1994, Olson and Owens 1998), set the precedent for other groups to apply these methods in other taxa, and to ask specific questions about the ecological, evolutionary, immunological, and behavioral relevance of carotenoid-specific color variation. Nearly 40 different carotenoids have now been described from a few hundred species spanning diverse avian families (reviewed in McGraw 2006b, LaFountain et al. 2015) and including several novel forms (LaFountain et al. 2010). With this large body of information, excellent

phylogenetic investigations have been undertaken to trace evolutionary patterns of carotenoid deposition, metabolism, and coloration (Prager and Andersson 2010; Friedman et al. 2014a,b). We now know, for example, that carotenoid pigmentation in plumage has evolved multiple times (as many as 13) across the avian orders, and that over 40% of families have species with carotenoid plumage coloration (Thomas et al. 2014a). Additionally, fine-scale chromatographic work on carotenoids in birds has permitted field ornithological investigations into the dietary limitations of particular carotenoids (McGraw 2006b) as well as physiological experiments on the role of specific carotenoids for boosting immunity (Fitze et al. 2007) or acquiring attractive plumage coloration (Saks et al. 2003).

The HPLC era has also stimulated the development of analytical methods for the two forms of melanin: eumelanin typically creates black and gray tones and pheomelanin typically creates buff and brown colors. This technique was created initially for analysis of mammal skin and hair (Ito and Wakamatsu 2003) and was co-opted for use with bird feathers (Haase et al. 1992, McGraw and Wakamatsu 2004). This preparation method specifically involves the degradation of the two melanin forms into products (pyrrole-2,3,5-tricarboxylic acid and 4-aminohydroxyphenylalanine, respectively) that are analyzed by HPLC (Ito and Wakamatsu 2003). By comparison to carotenoid analyses, these melanin characterizations have been performed on only a few dozen bird species (McGraw 2006a). But from what little has been done, we know that both eumelanin and pheomelanin are present in most melanic plumage colors (McGraw 2004, McGraw et al. 2004). Still, many researchers resort to inferring dominant melanin type from plumage color appearance (Galván and Møller 2013), and this suggests that HPLC techniques need to permeate more studies if we are to attain a deeper understanding of melanin plumage production and evolution at the biochemical level. Some new methods for quantifying melanin have also appeared in recent years (Zhou et al. 2012) that hold promise for more pervasive testing of melanin concentration in feathers, but these methods have only been tested in softer-tissued organisms to date, such as plants and insects (Debecker et al. 2015), and feathers may present a challenge to the extraction procedure.

In the last decade, we have also seen the HPLC-based identification of unique colorants in bird feathers, including both the unique aldehydes (psittacofulvins) that create the red and orange colors of parrot feathers (Stradi et al. 2001, McGraw and Nogare 2005) and the fluorescent, nitrogenous compounds that are responsible for the yellow and orange feathers of penguins (McGraw et al. 2007). HPLC has also recently improved our analyses of iris pterins and purines and facilitated investigation of, for example, sex and age differences in eye colorants of blackbirds (Hudon and Muir 1996). The same is true for eggshell porphyrin pigments as well, including the identification of biliverdin that creates blue and green shell coloration (reviewed in Gorchein et al. 2009).

Benefits and Challenges of Using Specimens

As with museum-based studies of avian morphology, demography, and evolution, for example, museum specimens provide a rich supply of biological material for analysis of pigments across the nearly full range of bird species. This is especially true for the colorants of plumage, as pigments appear to generally be preserved well in feathers of specimens that have been kept in the dark for extended periods of time. Unfortunately, as mentioned earlier for studies of coloration, bare-part colors fade over long periods of time, and this has notably limited our understanding of the distribution and evolution of avian bare-part pigmentation. In a few cases, in fact, pigments and coloration can fade in the feathers of museum skins as well (McNett and Marchetti 2005, Doucet and Hill 2009), so careful attention should be paid, if possible, to learning the preservation history of the skins being analyzed. An important question is, for example, have any specimens ever been used in exhibits and thus exposed to light for some period of time? Careful validation of the specimen colors/pigments under study with those seen in wild birds of the same species (Armenta et al. 2008a) is also critical.

Museum specimens can be precious or delicate, such that destructive sampling should be avoided if possible. Compared to spectrophotometric or photographic studies of coloration in museum specimens, pigment analyses typically incur a greater risk of specimen damage. At present, feathers from a bird skin must be trimmed

or plucked for full extraction and characterization of pigments with HPLC. Thus, scientists should be urged to carefully consider the goals of their pigment/coloration study while deciding the best course of action for pigment investigations using museum specimens.

Future Directions

Despite the many recent advances in biochemical analyses of avian integumentary pigments and associated studies of pigment diversity, mechanisms, and evolution in birds, much work still remains to be done. For example, we have not yet identified the integumentary pigments for the vast majority (>90%) of bird species; instead, pigment type has been inferred for a taxon based on reference specimen(s) within that lineage or from either visual estimation or spectral reflectance data (Thomas et al. 2013, Galván and Jorge 2015). Museum specimens could play a key role in a high-throughput pigment screening effort, until one can organize a large call for feather collection from all wild birds currently under study (Smith et al. 2003). For studies where feather removal from a skin is imperative, it may be useful for those preparing specimens to harvest feathers at the time of skinning and separately preserve these alongside the skin with as little modification to the integrity and appearance of the specimen, so that later plucking of feathers from aged/weathered specimens can be avoided. To improve studies of bare-part pigments in bird skins, it would be instructive to consider possible methods that one could employ at capture to preserve/characterize bare-part pigments.

Even among the select group of bird species in which integumentary pigments have been explored, there are many instances of incomplete characterization. The novel pigments in penguin feathers, the yellow psittacofulvins of parrots, and the fluorescent yellow in the down of game bird chicks still require comprehensive elucidation (McGraw et al. 2004). The pigments generating red and yellow plumage colors of adult game birds, such as the Golden and Lady Amherst Pheasant, have also proven particularly challenging analytically. Melanin is the most widespread colorant in the animal kingdom, and is present in all nonwhite structural colors of birds, yet we do not know how types and amounts of eumelanin and pheomelanin contribute to structural colors or have evolved across a wide range of birds. Last, chromatographically speaking, ultra-performance liquid chromatography (UPLC) has yet to be employed in bird pigment analyses and could enhance the detection of particular integumentary pigment types, for example, isomers and trace levels across Aves.

NONDESTRUCTIVE ANALYSIS WITH RAMAN SPECTROSCOPY

Plumage coloration is a rich source of ecological and evolutionary information and has been studied for many decades using relatively few analytical techniques. As described earlier, the two techniques most commonly used for feather color analysis are spectrophotometry and liquid chromatography, where spectrophotometry provides information about light absorption and reflectance properties (hue, brightness, and saturation), and liquid chromatography can provide deeper insight into pigment chemistry (Kritzler 1943, Dyck 1966). Absorbance and reflectance properties of feathers have proven valuable for analyzing bird health, social behavior, and other ecological and evolutionary parameters (Johnsen et al. 1998, Saks et al. 2003, Andersson and Prager 2006, Montgomerie 2006). Although spectrophotometry has the advantage of being a nondestructive technique (Montgomerie 2006), pigment identification has typically required destructive liquid chromatography analyses (Kritzler 1943, Stradi et al. 1995a). Indeed, liquid chromatography analyses on feather extracts have revealed an array of novel pigments (McGraw 2006b,c). Sample destruction is not always possible for museum specimens however; instead, pigment identification studies in ornithology collections increasingly use nondestructive Raman spectroscopy.

What Is Raman Spectroscopy?

Raman spectroscopy provides information about molecules and minerals in a sample by probing covalent bonds with a laser (Smith and Dent 2005). The instrumentation used for Raman spectroscopy can vary greatly, from a network of open-air mirrors and other equipment, to tiny components nestled inside a scanning electron microscope. More commonly, though, Raman spectroscopy is performed using a modified

binocular microscope, which allows for a very simple end-user experience. A sample is placed on the microscope stage and brought into the focal range of the microscope optics. Laser light is channeled through the microscope optics to the sample, and the light that scatters from the sample is channeled back through the optics toward a detector. Information from the detector is interpreted by a computer and used to calculate a Raman spectrum.

Scattered light is the essence of Raman spectroscopy. In brief, laser photons are first focused onto a sample; these photons interact with the sample by stimulating motion, that is, vibrations, between atoms that share covalent bonds. Energy is exchanged between the photons and the vibrating atoms. The photons scatter away from the sample and are channeled to a detector, allowing the energy that the photons have lost to the sample, or gained from the sample, to be calculated. The energy exchanged during the interaction between the laser photons and the sample is presented as a Raman spectrum, where each peak in the spectrum corresponds to a specific motion of covalently bound atoms. Peaks in Raman spectra are identifiable as components of a molecule or mineral, and chemically distinct structures have characteristic Raman spectra. Hence, Raman spectroscopy is useful for identifying distinct pigments. See Woodward (1967) and Smith and Dent (2005) for nonspecialist introductions to Raman spectroscopy, and Smith and Dent (2005) for a complete technical description with modern equipment.

Comparing Raman Spectroscopy and Spectrophotometry

Raman spectroscopy is still an unfamiliar technique to most avian biologists, whereas ultraviolet-visible spectroscopy (i.e., spectrophotometry) is commonly used. The following description explains the key differences between the two spectroscopy techniques (Figure 3.3).

Regarding spectrophotometry measurements, a light absorption spectrum is often presented as a series of intensity values against wavelength values. Each intensity value reveals the absorption of light at a particular wavelength, and light absorption spectra are intuitive to interpret as they correlate with the visual appearance of an object. A feather may appear orange because it absorbs blue

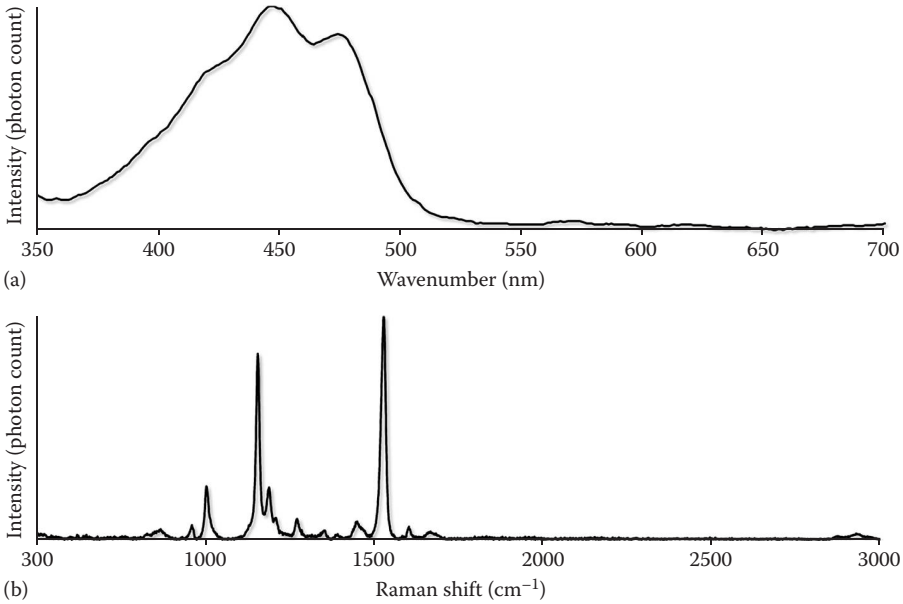

Figure 3.3. (a) UV-visible spectrum and (b) Raman spectrum from a feather pigmented with carotenoids. Spectrophotometry, also known as UV-visible spectroscopy, is routinely used to study the color of feathers and other tissues, whereas Raman spectroscopy is still comparatively rare in ornithology literature. Although spectra are the principal outputs of both spectroscopies, the data from each technique conveys fundamentally different information (see text).

wavelengths of light: a light absorption spectrum of an orange feather would have high intensity values in the blue wavelength range (450–495 nm). Spectrophotometry instruments can also measure reflectance spectra, which are essentially the inverse of an absorption spectrum. A reflectance spectrum of an orange feather would have high intensity values in the orange wavelength range (590–620 nm).

Unlike absorption or reflectance spectra, a Raman spectrum may not correlate with the visual appearance of an object. Instead, interpreting a Raman spectrum requires a moderate understanding of vibrational spectroscopy. A Raman spectrum is often presented as a series of intensity values against wavenumber values (note: not wavelength values), which are measurements of energy. A laser used in Raman spectroscopy will produce photons of a single color (e.g., green, 532 nm), and these photons will therefore all have the same energy value (18,797 wavenumbers, cm^{-1}). When photons interact with a sample they may lose or gain energy; the energy of the photon would become 17,277 cm^{-1} if the photon lost 1,520 cm^{-1} to the sample. The photon would lose 1,520 cm^{-1} if this is the energy "cost" of a vibrational mode within the sample (e.g., stretching of double bonds between carbon atoms). A Raman spectrum reports these energy gains or losses. The wavenumber scale in the Raman spectrum is presented as a Raman shift, where 0 cm^{-1} identifies the energy of the laser (i.e., no shift), and perhaps counterintuitively, positive cm^{-1} values describe energy losses to the sample. Most Raman spectra of molecules have multiple peaks, corresponding to multiple vibrational modes within the molecule. The positions of these peaks are often used as a "chemical fingerprint" for identifying a molecule.

AN OVERVIEW OF RAMAN SPECTROSCOPY AND PLUMAGE PIGMENTS

In the first study to use Raman spectroscopic measurements of pigmented feathers, Stradi et al. (1995b) examined carotenoid pigments in the plumages of eight cardueline finch species, mostly to demonstrate a new method of carotenoid extraction. However, the authors also were interested in the link between the type of carotenoids present in feathers and perceived coloration, and showed that the red and yellow plumage patches of European Goldfinches (*Carduelis carduelis*) contained the same types of carotenoids. Important color information that is lost during pigment extraction was recovered by studying the pigments *in situ* with Raman spectroscopy (Stradi et al. 1995b). Subtly different Raman spectra were recorded from the red and yellow plumage patches, which both contained canary xanthophyll A and canary xanthophyll B. The Raman spectra from each patch had peaks expected for carotenoids, but the peak positions differed between patches. Stradi et al. (1995b) proposed that the difference in Raman spectra could be explained by "considering the mode of attachment of the carotenoids to different keratin structures." When bound into feathers, the canary xanthophyll molecules likely adopted different conformations, which altered their perceived coloration. Raman spectroscopy is well suited for studying avian pigments *in situ* to understand the relationship between pigment chemistry and plumage color (Stradi et al. 1995b).

"Color-tuning" in plumages refers to chemical interactions that alter the electronic structure of a pigment molecule and therefore change the perceived color. Raman spectroscopy helped reveal that color-tuning could explain the variation in color between red feathers from Scarlet Ibis (*Eudocimus ruber*), orange-red feathers from Summer Tanager (*Piranga rubra*), and violet-purple feathers from White-browed Purpletuft (*Iodopleura isabellae*) (Mendes-Pinto et al. 2012). The same carotenoid (canthaxanthin) was present in the plumages of each bird, and shifts in Raman spectral peaks showed that the pigments were held in subtly different molecular configurations. Likewise, Berg et al. (2013) used Raman spectral data to propose that the interaction between multiple carotenoid molecules (i.e., exciton coupling between chromophores) was a potential explanation for the color variation from "brilliant red to magenta or purple" across rhodoxanthin-pigmented plumages. Color differences between the plumages of two broadbill species studied with Raman spectroscopy (Black-and-red Broadbill, *Cymbirynchus macrorhynchos*; Banded Broadbill, *Eurylaimus javanicus*) has also been attributed to color-tuning: "the polarizing influence of charges nearby the carotenoid, hydrogen bonding, or possibly exciton coupling among neighboring chromophores" (Prum et al. 2014). Although the exact color-tuning mechanism for plumage pigments is still elusive, nondestructive Raman spectroscopy has

provided fundamental data that will guide its eventual discovery.

Beyond carotenoids and color-tuning, a diverse range of avian pigments has been analyzed with Raman spectroscopy. New insights into the structures of parrot-specific and penguin-specific pigments have been revealed through *in situ* analyses, and experiments with biologically ubiquitous melanins have also been reported (Veronelli et al. 1995; Galván et al. 2013a,b; Thomas et al. 2013; Galván and Jorge 2015). Raman spectroscopy has proven to be a useful technique for pigment surveys in ornithology collections.

Applications of Raman Spectroscopy in Museum Collections

Raman spectroscopy was applied to specimens from the National Museum of Natural History, Smithsonian Institution to show that a nondestructive technique could predict the most abundant type of carotenoid in a feather (Thomas et al. 2014b). Fossil feathers preserved in amber were studied with a confocal Raman microscopy instrument in a search for ancient pigments (Thomas et al. 2014c). Raman spectroscopy was also used to expand the known taxonomic distribution of carotenoid plumage pigments (Thomas et al. 2014a). These and other recent reports show the potential of Raman spectroscopy for studies of the specimens found in ornithology collections. Two studies in particular are good platforms for future collections-based research, the color-tuning investigation of Berg et al. (2013), and the carotenoid-type analyses of Thomas et al. (2014b).

Plumage color-tuning mechanisms that involve carotenoid pigments are largely unexplored (Shawkey and Hill 2005). Berg et al. (2013) sought evidence for a color-tuning mechanism in feathers pigmented with the carotenoid rhodoxanthin and presented a set of viable candidates. Spectra in Berg et al. (2013) may help subsequent researchers to find Raman spectral evidence for a particular tuning mechanism, allowing fine-scale selection pressures on plumage color to be studied. Consider that, in addition to the costs of accumulating and displaying carotenoids, some birds invest additional resources to achieve a narrow hue, brightness, and saturation range. The importance of color tuning is well established in many other plant and animal systems (Björn and Ghiradella 2015), and if evidence of a color-tuning mechanism can be discovered in plumage spectra, then it would be possible to study the prevalence of this trait among birds.

Thomas et al. (2014b) also studied Raman spectra collected from carotenoid-pigmented plumage. Carotenoids have a distinctive Raman spectrum that contains three principal peaks that vary slightly in position and intensity for different carotenoids (Veronelli et al. 1995, Thomas et al. 2014b). Raman spectroscopy could therefore be used to taxonomically map plumage carotenoids (Thomas et al. 2014a), revealing associations between lineages and particular types of carotenoids (e.g., Mendes-Pinto et al. 2012).

Raman spectroscopy occupies a valuable analytical niche for pigment research. Like spectrophotometry, Raman spectroscopy is nondestructive and requires no specialized sample preparation. Like liquid chromatography, Raman spectroscopy can be used to chemically identify pigments. The use of Raman spectroscopy for plumage studies has surged recently as researchers have begun to explore the potential of this technique. However, Raman spectroscopy is a potentially valuable technique for all pigmented tissues, not just plumage, and will likely see wider application in coming years.

ADVANCES IN STUDYING STRUCTURAL COLORATION

Avian coloration is produced by a combination of two main mechanisms. The first is the absorption of particular wavelengths of light by pigmentary molecules and analyses of such pigments were described earlier. The second is the differential reflection and refraction of light by biological materials, such as pigmentary and keratin molecules, which is generally referred to as "structural color" (Prum 2006). Structure and pigments cannot operate independently—structural color needs biological molecules like pigments to scatter light (Prum 2006, Shawkey and Hill 2006), and pigmentary color needs structure to reflect the wavelengths of light that are not absorbed (Prum 2006, Shawkey and Hill 2006). However, we can define pigmentary color as color whose reflective properties (e.g., hue) depend primarily on the wavelengths of light not absorbed by a pigment molecule and structural color as color whose reflective properties depend primarily on the light being reflected or refracted by the nanostructures

present in the biological material (Prum 2006). Note that it is possible for a biological structure to be made up of the interaction of pigmentary and structural color, as in the case of the plumage of the Budgerigar (*Melopsittacus undulatus*), whose green feathers are a combination of yellow caused by pigments and blue caused by structure (D'Alba et al. 2012). Structural color can be present in avian facial skin, bills, legs, irises, and plumage coloration (Prum and Torres 2003a, Prum 2006).

Mechanisms of Structural Coloration

The mechanisms for the production of structural color can be broadly divided into two types: incoherent and coherent scattering (Prum 2006). Incoherent scattering is characterized by randomly scattered wavelengths of light and is the mechanism behind white, unpigmented feathers (Prum 1999, 2006). For coherent scattering, the light-scattering biological molecules are not randomly distributed, and the wavelengths of light constructively interfere to produce particular colors (Prum et al. 1998; Prum 1999; Prum and Torres 2003a,b), including many of the greens, blues, violet, and ultraviolet colors observed in avian plumage and integumentary structures (Prum 2006; Prum et al. 1998, 2003; Prum and Torres 2003a; Stoddard and Prum 2011). Coherent scattering can be present either in the barbule, producing iridescence, or in the feather barb, generally producing noniridescent colors (Prum et al. 1998, Prum and Torres 2003a, Prum 2006). Within feather barbules, arrays of melanosomes can be arranged in single layers or multilayer crystal-like structures (Prum 2006) and shift the angle of light incidence to produce shifts in color properties, such as hue (Osorio and Ham 2002), which is termed iridescence. One common form of iridescence, that seen on the oil slick-like dark plumage of iridescent members of Icteridae, can be described by thin-film modeling (Shawkey et al. 2006a, Maia et al. 2009), and is produced by a thick layer of keratin on top of a single layer of melanin molecules (Prum 2006). Alternatively, in multilayer arrays, hollow or solid melanosomes can also be arranged in stacks to produce the bright colors such as those observed in hummingbirds (reviewed in Prum 2006).

Noniridescent colors produced in feather barbs are created by quasi-ordered arrays of keratin and air located below the cortex of the feather barb (Prum and Torres 2003a, Prum 2006). The shape of these air-filled channels can be either sphere-like or channel-like (Prum and Torres 2003a, Saranathan et al. 2012), but are uniform in shape and size within a feather barb (Prum and Torres 2003a). The uniform shape of these air-filled channels dictates which wavelength of light will be scattered by any given structure, but, unlike iridescence, the hue will not change with viewing angle (Prum and Torres 2003a). While these arrays produce colors from their physical properties alone, the scattered light can also be partially absorbed by pigments (such as carotenoids or psittacofulvins) that are present in the cortex. This combination of feather structure and pigments can produce hues not created by either alone (D'Alba et al. 2012).

Techniques to Describe Structural Coloration

The colors produced by structural mechanisms can be studied using the photographic or spectrophotometric methods described in previous and subsequent sections. It is also possible to investigate the contributions of different mechanisms to the observed color. To do this, one could remove underlying pigments, like carotenoids, and measure the structure alone (Shawkey and Hill 2005, Jacot et al. 2010). Alternatively, one can saturate feathers in a substance like Cresol (Sigma, St. Louis, MO) that has the same refractive index as keratin to disrupt structural color and measure the pigmentary color alone (Shawkey and Hill 2005).

Additional techniques can be applied to museum specimens to describe the underlying physical structures that produce structural coloration (reviewed by Vukusic and Stavenga 2009). One of the most common techniques is transmission electron microscopy, which can be used to describe the internal nanostructure of feather barbs or barbules (Prum 2006). In the case of iridescent color, the measurements from these images can be applied to single or multilayer thin film models (Prum 2006, Vukusic and Stavenga 2009). The resulting two-dimensional images from quasi-ordered arrays, like those that produce noniridescent structural color in birds, can be described by a Fourier transformation and can predict the shape of the resulting reflectance spectrum (Prum et al. 1998, Prum and Torres 2003a). For example, to obtain a three-dimensional

reconstruction of the feather from an Eastern Bluebird (*Sialia sialis*), Shawkey et al. (2009) applied intermediate voltage electron microscopy and more accurately modeled the quasi-ordered structure with a three-dimensional Fourier analysis. Finally, small-angle x-ray scattering can also be used to describe the quasi-ordered structure of noniridescent structural colors (Saranathan et al. 2012).

Applications to Ecology and Evolutionary Biology

Methods used to quantify the nanostructure responsible for the production of structural coloration can be applied to studies of ecology and evolutionary biology. When coupled with museum specimens, these methods have been used to study the anatomical basis for sexual dichromatism (e.g., Shawkey et al. 2005), the mechanism for geographic plumage color differences within a species (e.g., Doucet et al. 2004), and the evolution of iridescent plumage (e.g., Shawkey et al. 2006b) and complex nanostructures (Eliason et al. 2015). Nonetheless, this area remains rife with opportunity.

One study, highlighted here, was conducted by Maia et al. (2013b), and combined transmission electron microscopy, spectrophotometry, and powerful phylogenetic comparative methods to describe how melanosome morphology influenced diversification within the African starlings (Sturnidae). The authors used transmission electron microscopy on feathers from museum specimens to confirm previously described melanosome morphology in at least one species per genus, or more where species were reported to have different morphologies than their closest relatives. They measured reflectance spectra from males and females and analyzed these spectra using the avian color space model (Stoddard and Prum 2008). They then reconstructed the ancestral state of melanosome morphology using reversible-jump Markov chain Monte Carlo, identified the best model of color evolution by comparing models of random evolution (Brownian motion), stabilizing selection (Ornstein-Uhlenbeck processes), or combinations of these models with melanosome type, and estimated diversification rates within the clade. They found that the simple, rod-shaped melanosomes were the ancestral melanosome morphology in the clade, that these melanosomes repeatedly evolved into the more complex morphologies, and that the evolution of these more complex melanosome morphologies not only allowed for a broader area of color space, but that they accelerated the evolution of color differences in coloration between species. Finally, the authors showed that lineages with the more complex melanosome morphologies had faster diversification rates, which has implications for the influence of social signals on lineage diversification. This is just one example of what can be learned by studying a large number of species, an area that is also rife with opportunity, and illustrates the type of project that is greatly facilitated by using the rich resource available from museum skin specimens.

Structural Coloration in Fossil Feathers

While most of what we know about plumage coloration in birds comes from extant species, melanosomes or other pigment molecules can be preserved in fossils. These fossilized molecules can be used to infer likely coloration patterns, including structural coloration (Vinther et al. 2010, Li et al. 2012, Vinther 2015). Structural coloration in fossils was first described in the context of melanosome distribution and morphology to differentiate between eumelanin and pheomelanin (Vinther et al. 2008). In many fossil feathers, the beta-keratin is degraded (Vinther et al. 2010), and so the ability to reconstruct noniridescent structural colors in feather barbs that rely on the organization of keratin and air molecules is limited (Vinther 2015), at least at present. However, the organization of the melanosomes within the barbules can be well-preserved in some cases, and similarities to extant species suggests that some feathers displayed iridescent structural color (Vinther et al. 2010). Using this approach, Li et al. (2012) hypothesized that the feathered dinosaur *Microraptor* likely had predominantly iridescent plumage. The extent of structural coloration is still unknown in early birds and dinosaurs, but is likely to expand in breadth as researchers examine and discover additional well-preserved fossils.

Future Directions

Knowledge of structural coloration has increased dramatically in the last 15 years, but remains a topic open for exploration and study. It will only be through broad surveys of species throughout

the avian tree of life, coupled with the ever-increasing knowledge of their genetic relatedness, that we will be able to understand how these mechanisms evolve and to correlate them with life history traits. Museum collections provide a rich resource for completing these surveys by providing the material to broadly sample species throughout the avian tree. Together with spectrophotometer measurements and pigment information, these studies will provide insights into how the gamut of avian coloration evolves in concert with the underlying coloration mechanisms.

Intraspecific differences in feather structure have rarely been studied, but could provide essential information as to how structural coloration might vary within or between populations or individuals. Museum collections also provide a rich resource of material for these types of studies, and can even be used to examine how feather structure might change in a population in historical time.

Finally, many recent advances in the study of structural coloration have come about by collaborations between biologists and physicists. It is only by combining expertise in these areas can we fully understand the basis of these mechanisms and discover previously unknown types of structural coloration.

ADVANCED METHODS FOR STUDYING AVIAN EGG COLOR

With their striking variation in color and pattern, avian eggs provide a compelling system for investigating the mechanisms and functions of animal coloration. Although they historically have received less research attention than skin collections, egg collections provide a valuable record of life history and behavior, and a rich reservoir of material for researchers (Scharlemann 2001, Kiff and Zink 2005). Consider the collection of eggs at the Natural History Museum in Tring, United Kingdom, which houses over 300,000 clutches and is one of the most comprehensive and actively used egg collections in the world. In recent years, this egg collection has provided the raw material for discoveries related to pigment chemistry (Cassey et al. 2012a), ultraviolet light exposure and solar radiation (Maurer et al. 2015), signal diversity (Cassey et al. 2012b), camouflage (Hanley et al. 2013), egg mimicry (Stoddard and Stevens 2010, 2011), and egg pattern signatures (Stoddard et al. 2014). New tools for quantifying coloration have helped to usher in a new era of research on avian eggs. Here we briefly introduce the basics of egg coloration and then describe the four main techniques used in museum-based studies—chemical analysis, structural analysis, spectrophotometry, and digital photography—all of which have parallels to the study of plumage and skin coloration.

Egg Coloration: An Overview

The full range of egg coloration appears to stem from just two tetrapyrrole pigments: a red-brown pigment called protoporphyrin and a blue-green pigment called biliverdin (Kennedy and Vevers 1976, McGraw 2006c, Gorchein et al. 2009, Sparks 2011). Both pigments are involved in the biosynthesis of heme, an iron-containing compound important for oxygen transport in the blood stream of vertebrates (Baird et al. 1975). Pigments are deposited on eggshell in the shell gland during the final stages of egg formation, with the bulk of the pigment distributed in the cuticle, an organic layer that typically coats the shell (Hincke et al. 2012). While evidence suggests that biliverdin is produced *de novo* in the shell gland, it is not clear whether the same is true for protoporphyrin, which may be synthesized elsewhere in the body and subsequently mobilized to the shell gland (Sparks 2011). It is also important to note that eggshells that appear white do not necessarily lack pigment, as sometimes protoporphyrin and biliverdin are detected even in white shells (Kennedy and Vevers 1976, Sparks 2011). The glossiness of eggshells, most evident in the highly reflective sheen of many tinamou eggs, results from an extremely smooth cuticle that modifies the appearance of the underlying background color (Igic et al. 2015).

From an evolutionary standpoint, the ancestral egg type was probably white (reviewed in Kilner 2006). However, the detection of both tetrapyrrole pigments in many ratite eggs, including in extinct moa species (Igic et al. 2009), suggests that egg pigments evolved early in birds and are likely to be highly conserved throughout avian evolution. Why are bird eggs colorful? A suite of selective forces likely influences egg appearance, including camouflage, brood parasitism, sexual signaling, thermoregulation, antimicrobial defense, embryonic development, and eggshell strength.

The evolutionary patterns and ecological functions of egg coloration have been reviewed extensively (Underwood and Sealy 2002, Kilner 2006, Reynolds et al. 2009, Cherry and Gosler 2010, Cassey et al. 2011, Maurer et al. 2011, Stoddard et al. 2011, Hauber 2014). We direct readers to the reviews listed here for detailed information, as here we focus on the analysis of egg coloration using museum collections.

Chemical Analysis

As with feather pigments, high-performance liquid chromatography (HPLC) combined with mass spectrometry (MS) is a common technique used for the analysis of eggshell pigments (reviewed in Gorchein et al. 2009). HPLC (usually reverse phase-HPLC) provides a mechanism for separating chemicals along a column; these chemicals are then detected by a mass spectrometer, which provides information about the chemical component's mass and identity. Detailed information about HPLC-MS techniques specific to porphyrins can be found in Lim (2004). With respect to eggshell pigment extraction, researchers typically follow procedures outlined by Mikšík et al. (1996), which involve cutting a small fragment of eggshell and dissolving it in a solution containing methanol and sulfuric acid (Cassey et al. 2012a,b). Some caution should be exercised here, as there are drawbacks to using a methanolic sulfuric acid solution for pigment extraction (Gorchein et al. 2009). However, drawbacks such as the unsuitability of the method for detection of metal compounds may be irrelevant if metal-containing compounds are truly absent from eggshell pigments, which—contrary to the findings of early studies (Kennedy and Vevers 1976)—appears to be the case (Gorchein et al. 2009). Using HPLC-MS, extracted pigments are then identified by comparison to commercially sourced standards, and their concentrations are quantified. Pigment concentration must then be standardized relative to the eggshell fragment's mass or surface area, depending on how the pigment is distributed throughout the shell (Cassey et al. 2012a).

Recent studies on egg pigment chemistry have made exciting contributions to research on breeding behavior, extinct species, and brood parasite-host dynamics. For example, in a broad survey of eggshell pigment concentrations across different groups of British birds, Cassey et al. (2012b) found that, while controlling for phylogenetic relatedness, pigment concentrations are correlated with different ecological and life-history strategies. Specifically, high levels of protoporphyrin are associated with cavity nesting and ground nesting, while biliverdin concentration is associated with noncavity nesting and a greater likelihood of biparental care. Researchers have also demonstrated that eggshell pigments can be extracted from eggshells that are over 500 years old; using HPLC-MS, Igic et al. (2009) successfully recovered biliverdin and protoporphyrin from various extinct species of moa. Finally, comparing the pigment composition of eggshells laid by the brood parasitic Common Cuckoo and its hosts has revealed that cuckoos and their hosts color their eggs using the same pigments and in similar concentrations (Igic et al. 2012).

Chemical analysis of eggshell pigments also holds promise for the study of pesticides and other environmental contaminants. Classic studies exploring the effect of pesticide contamination on eggshells focused on measures of shell thickness (Hickey and Anderson 1968). However, two recent studies suggest that egg appearance itself may be indicative of contaminant load, such that some aspects of egg coloration might provide a rapid and nondestructive means of assessing local pesticide levels (Jagannath et al. 2007, Hanley and Doucet 2012). Though these studies report correlations between measures of egg appearance such as blue-green coloration and pesticide load, neither study quantified pigment concentration. Relating pigment concentration to contaminant load is an important next step for elucidating the mechanisms by which contaminants influence egg coloration. Note that estimates of pigment concentration cannot be made reliably from visual assessment or photographs, at least in some cases (Brulez et al. 2014). For this reason, chemical analyses of eggshell tend to be destructive. Consequently, "shoebox" collections (Russell et al. 2010), which typically lack the high-quality data required for the main collection, are of particular value to researchers, and museum curators should be encouraged to accept such material when the opportunity is presented. Moving forward, it will be important to develop chemical analysis methods that minimize damage to specimens. Promisingly, Raman spectroscopy, a nondestructive spectroscopic method that requires no specialized sample preparation (see earlier

section "Nondestructive Analysis with Raman Spectroscopy"), was recently used to detect biliverdin and protoporphyrin from eggshell specimens (Thomas et al. 2015). However, pigment composition may be difficult to determine if both pigments are present in the eggshell, and it is not yet clear whether Raman spectroscopy can provide reliable information about pigment concentration (Thomas et al. 2015).

Structural Analysis

Several nonsignaling hypotheses for the diversity of egg coloration require a detailed understanding of eggshell strength and ultrastructure. Following the initial suggestion from Solomon (1987) that protoporphyrin pigment might contribute to eggshell strength, Gosler et al. (2005) proposed the "structural function" hypothesis for protoporphyrin pigmentation. The hypothesis posits that birds might add pigment to the shells to compensate for shell thinning, which arises from calcium deficiency, and that the added pigment, in turn, affects the egg's mechanical and water vapor conductance properties. Correlative evidence for the hypothesis has been mixed (Gosler et al. 2011, Bulla et al. 2012, Mägi et al. 2012). Another suite of hypotheses suggests that egg pigments interact with the light environment to affect embryonic development (Lahti 2008, Cassey et al. 2011, Maurer et al. 2011). The main ideas here are that shell pigments may protect the developing embryo from overheating, block harmful radiation, provide antimicrobial defense, and serve as wavelength-specific filters, creating a particular light environment that may influence the speed of embryonic growth (Maurer et al. 2011, Maurer et al. 2015).

To fully address these nonsignaling hypotheses, researchers must employ a range of advanced techniques to describe eggshell structure, thickness, strength, water vapor conductance, and light transmission. For analyzing eggshell structure, scanning electron microscopy (SEM) is often used to capture details of the shell's surface texture or of its interior structure (Hincke et al. 2012). Energy-dispersive x-ray spectroscopy (EDS), which provides information about the elemental composition of different eggshell layers, can serve as a useful complement to SEM imaging (Igic et al. 2011). Thickness measurements are typically obtained by a micrometer (Maurer et al. 2012) or from SEM images; the techniques yield similar results (Igic et al. 2010). The mechanical properties of eggshell are commonly assessed using a range of methods, including Vickers hardness testers (Igic et al. 2011) and Instron universal testing frames (Gosler et al. 2011). Water vapor conductance is typically measured by mounting eggshell fragments on test tubes, placing them in a desiccator, and then measuring mass loss, where mass loss is assumed to be water vapor escaping out of the shell (Portugal et al. 2010b). Finally, light transmission is measured using a spectrophotometer, which measures the light that passes through eggshell fragments mounted on plastic cuvettes (Maurer et al. 2015). A goal for future work will be to incorporate these techniques into rigorous, multifaceted studies that address multiple hypotheses about the function of structural variation in eggshells (e.g., Maurer et al. 2015). As with chemical analysis, structural analysis of eggshell material is typically destructive. One exception is the measurement of eggshell thickness, which can sometimes be assessed with measurements through the shell's blowhole or predicted from shell mass and dimensions (see Maurer et al. 2012).

Spectrophotometry

As with feathers, the adoption and then widespread use of spectrophotometry (Andersson and Prager 2006) helped to revolutionize the study of egg coloration. Researchers interested in egg mimicry were quick to use spectrophotometry, demonstrating that cuckoos mimic host eggs across the bird-visible range of wavelengths (Cherry and Bennett 2001, Avilés et al. 2006, Starling et al. 2006). Similarly, many researchers testing the sexually selected eggshell coloration hypothesis (Moreno and Osorno 2003) employed spectrophotometry (reviewed in Reynolds et al. 2009). In these earlier studies, researchers typically used the raw spectra to extract colorimetric variables or to perform PCA (Montgomerie 2006). However, the recent trend has been toward incorporating visual models, which are combined with the raw spectral data to provide estimates of retinal quantal cone catch (see "Analytical Approaches" under "Spectrophotometry" section). In studies that make explicit predictions about signaling (e.g., mimicry, camouflage, sexual signaling, communal breeding), it is important to apply a visual

model that is relevant to the signal receiver, which is usually another bird. This approach has been embraced in diverse studies of egg coloration in the field (Spottiswoode and Stevens 2010, Yang et al. 2013) and in museum collections (Stoddard and Stevens 2011, Hanley et al. 2013, Abernathy and Peer 2014), including a broad comparative assessment of egg color variability in museum eggshells representing 251 species (Cassey et al. 2010a). A comprehensive review of avian vision and its application to the study of egg coloration can be found in Stevens (2011), which provides detailed recommendations about spectrophotometry, digital photography, and visual modeling (also see upcoming section "Case Study").

When obtaining spectral data from eggs, there are a few special points to consider. First, it can be challenging to obtain reliable spectra across the egg, especially for maculated (speckled) parts of eggs because speckles can be very small. To help account for speckling, it is advisable to use a custom narrow-ended (1/8-inch diameter) probe (available from Ocean Optics, Dunedin, FL); hold the probe at a constant distance and a fixed angle to the egg surface; and measure reflectance at the top, middle, and base of the egg. Where possible, separate measures should be obtained from the egg background and speckled portions of the egg (Stoddard and Stevens 2011). Second, to avoid contaminated spectra, which overlap with neighboring colors, consult Akkaynak (2014). Third, egg colors can change over time in museum collections (Starling et al. 2006; Cassey et al. 2010b, 2012c), so it is important to consider storage duration, age of the specimen, and measurement device when analyzing egg colors. Recently, Navarro and Lahti (2014) determined that blue-green eggshells decreased in overall reflectance and shifted slightly in terms of spectral shape when exposed to broad-spectrum light under lab conditions for several days; however, these differences happen gradually and extensive handling and exposure of museum eggs would be required to produce large errors. More work on egg fading and photodegradation is needed, particularly for a broader range of egg colors as blue-green egg colors have been the focus so far. Finally, it is worth remembering that egg collections do not always present a random cross-section of wild eggs because some collectors may favor unusual eggs, such as excellent mimics (Starling et al. 2006). Using large sample sizes of clutches from diverse localities, and those acquired by different collectors, can help to counteract these sources of bias and to prevent pseudoreplication.

Digital Photography

Digital photography is especially useful for the study of avian eggs because point-by-point spectrophotometry does not capture the spatial arrangement of egg patterning, which is an important aspect of egg coloration in many avian species (Kilner 2006, Hauber 2014). Already, digital photography, combined with novel ways of quantifying spatial patterns, is helping to shape our understanding of egg camouflage (Lovell et al. 2013), egg mimicry (Spottiswoode and Stevens 2010, Stoddard and Stevens 2010), and egg signatures (Stoddard et al. 2014, Caves et al. 2015). We refer the reader to the earlier section on digital photography and to the following case study for further details.

Case Study

Brood parasites sneak their eggs into the nests of other species, off-loading all parental care to host birds (Davies 2000). The cost of parasitism often triggers an evolutionary arms race between brood parasites and their hosts, with hosts evolving shrewder defenses and parasites evolving better tricks, such as egg mimicry (Rothstein 1990). To study egg mimicry by the Common Cuckoo, Stoddard and Stevens (2010) combined digital image analysis and a model of avian luminance vision with a recently developed method of quantifying spatial patterns called "granularity analysis" (see Figure 3.4b). They found that cuckoo host-races have evolved better egg pattern mimicry for those host species showing the strongest egg rejection. Stoddard and Stevens (2011) next used reflectance spectra to analyze egg color mimicry by the cuckoo. They applied two models of avian color vision to test for egg mimicry: the Vorobyev-Osorio receptor noise-limited discrimination model (Vorobyev and Osorio 1998), which can be used to calculate threshold differences between two colors, and the avian tetrahedral color space model (Goldsmith 1990, Endler and Mielke 2005, Stoddard and Prum 2008), which can be used to represent colors in a three-dimensional chromaticity diagram based on avian spectral sensitivities (Figure 3.4a). Both visual models

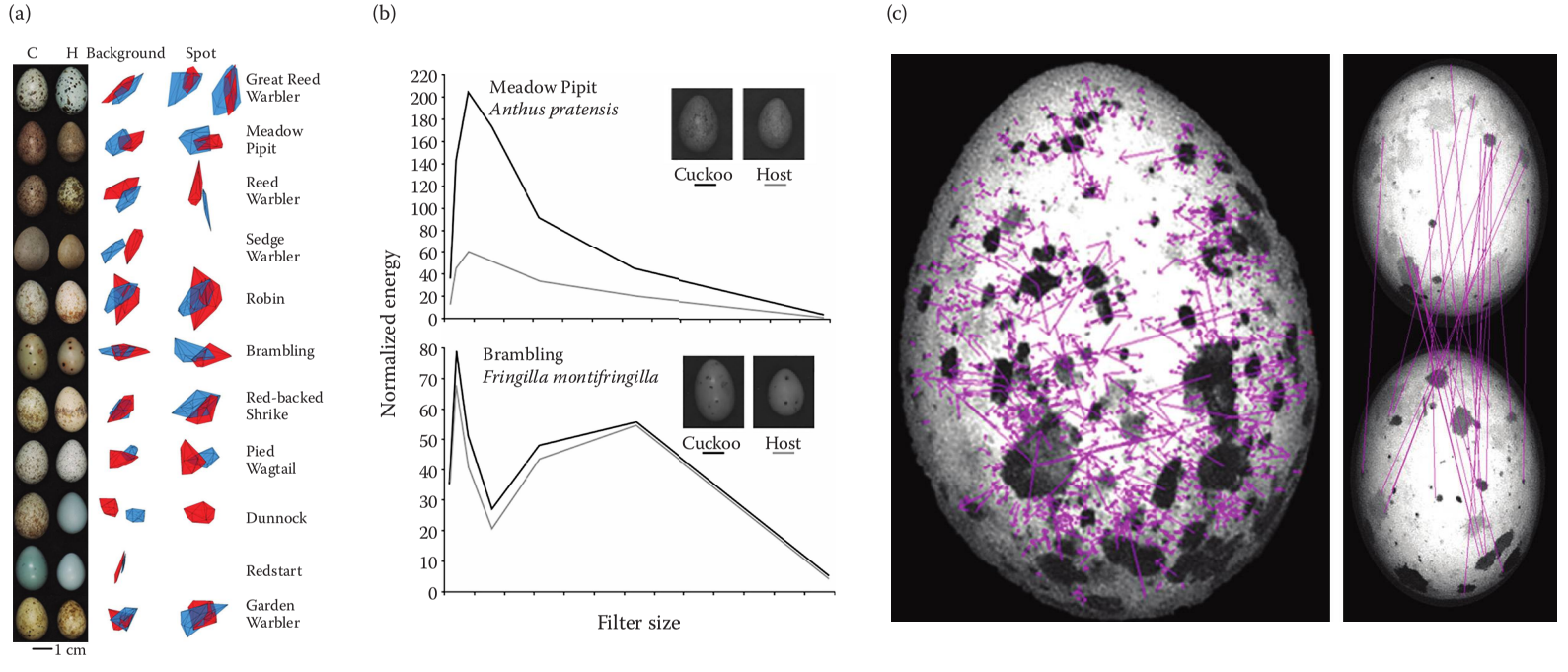

Figure 3.4. New methods for analyzing avian egg color and patterns, applied to cuckoo–host coevolution. (a) Photographs of Common Cuckoo (left) and host (right) eggs are shown next to the color distributions of egg background and spot colors in avian tetrahedral color space, which accounts for the fact that birds have four color cones in their retinas. This illustrates the extent of overlap between cuckoos (red) and their target hosts (blue). The overlap can be used as an estimate of egg color mimicry from a bird's-eye view. (Reproduced with permission from Stoddard and Stevens, 2011. Photographs within the figure are copyright of the Natural History Museum, London [NHM], and the University Museum of Zoology, Cambridge [UMZC].) (b) Average granularity spectra for two hosts (Meadow Pipit and Brambling) and their respective cuckoo gens, or host-race, showing the contribution of different marking sizes to the overall egg pattern. Where the granularity spectra are a good match (as in the Brambling and its cuckoo), the cuckoo egg pattern matches many aspects of the host's egg pattern. (Reproduced with permission from Stoddard and Stevens, 2010. Photographs within the figure are copyright of the NHM.) (c) To study the recognizability of egg patterns, individual features are extracted from a host egg using the SIFT algorithm (left). (Reproduced with permission from Stoddard et al., 2014.) A novel pattern matching algorithm, NaturePatternMatch, then searches for matching features on pairs of eggs, like the pair shown here, laid by the same female (right). For details, see Stoddard et al. (2014). (Photographs within the figure are copyright of the NHM.)

confirmed that cuckoo host-races have evolved better egg color mimicry for highly discriminating hosts. Finally, Stoddard et al. (2014) used digital images and a new pattern recognition algorithm (NaturePatternMatch) to demonstrate that host birds have fought back against cuckoo mimicry by evolving individually recognizable patterns on their own eggs (Figure 3.4c). All three of these studies were performed on eggs held in museum collections. Spectrophotometry, digital photography, and advanced models of avian vision and recognition provide outstanding avenues for studying egg coloration in new contexts.

Future Directions

Researchers are now poised to pursue questions about egg coloration with unprecedented rigor and creativity. Museum egg collections will continue to play a vital role in facilitating this work. In the future, it will be fascinating to see how the study of egg coloration will be influenced by new technical advances occurring within museum egg collections, particularly with respect to DNA extraction and whole genome amplification (Lee and Prys-Jones 2008), proteomic analysis (Portugal et al. 2010a), fossil ancient DNA (Oskam et al. 2010), and pollution (Ruuskanen et al. 2013). The critical importance of maintaining and bolstering egg collections cannot be overstated, particularly for use in future studies investigating long-term changes in bird populations that are reflected in eggs (Scharlemann 2001, Kiff and Zink 2005). Finally, museums should be strongly encouraged to digitize their egg collections using photographs. Photographs can be efficiently obtained and easily stored online, and they provide key information about color, pattern, and egg morphology. Care should be taken to use calibrated, UV-sensitive digital cameras, controlled light sources, and appropriate color standards in all images.

CONCLUSIONS

These are exciting times to be studying avian coloration. Just a few decades ago, studies of avian color were mostly limited to human-based assessments of plumage color or labor-intensive biochemical analyses. As outlined in this chapter, recent advances have dramatically expanded the types and nature of approaches that can be used. These advances include quantifying avian coloration using spectrophotometry, high-performance liquid chromatography, new methods for assessing structural color, Raman spectrophotometry, and digital photography and associated analytical techniques. As a result, researchers can now assess coloration from an avian visual perspective, and detailed information on the pigments and structure underlying coloration is known for many species. In addition, these advances have facilitated a better understanding of egg coloration. The growth of data on avian color has greatly expanded our overall understanding of the evolution and ecology of birds. Future directions include more advanced digital photography, the potential to uncover color information from fossils, and more precise assessment of pigments. And how the same pigments can result in different colors. All of these approaches have expanded the role of the traditional study skin, revealing data not typically visible to the naked eye. Thus, they illustrate how the concept of the "extended specimen" is expanding the use of museum collections for the study of avian coloration.

Museum collections represent archives of nature's variation, including variation in color across time and space. These collections allow researchers access to extinct species and extinct populations as well as rare species that cannot be easily sampled in the wild. In addition, they allow researchers to survey densely across taxonomic groups, something that would be prohibitively expensive if all species needed to be sampled in nature. Furthermore, specimens in museum collections preserve the coloration of past populations, allowing for the study of adaptation in coloration across time. For all these reasons, museum collections will continue to provide the essential foundation on which future studies of avian color can be based. These future studies will likely extend the use of specimens even beyond the approaches outlined here.

LITERATURE CITED

Abernathy, V. E., and B. D. Peer. 2014. Intraclutch variation in egg appearance of Brown-headed Cowbird hosts. Auk 131:467–475.

Akkaynak, D. 2014. Use of spectroscopy for assessment of color discrimination in animal vision. Journal of the Optical Society of America A 31:A27–A33.

Akkaynak, D., T. Treibitz, B. Xiao, U. A. Gürkan, J. J. Allen, U. Demirci, and R. T. Hanlon. 2014. Use of commercial off-the-shelf digital cameras for scientific data acquisition and scene-specific color calibration. Journal of the Optical Society of America A 31:312–321.

Andersson, S., J. Örnborg, and M. Andersson. 1998. Ultraviolet sexual dimorphism and assortative mating in blue tits. Proceedings of the Royal Society B 265:445–450.

Andersson, S., and M. Prager. 2006. Quantifying colors. Pp. 41–89 in G. E. Hill and K. J. McGraw (editors), Bird coloration volume 1: mechanisms and measurements, Harvard University Press, Cambridge, MA.

Armenta, J. K., P. O. Dunn, and L. A. Whittingham. 2008a. Effects of specimen age on plumage color. Auk 125:803–808.

Armenta, J. K., P. O. Dunn, and L. A. Whittingham. 2008b. Quantifying avian sexual dichromatism: A comparison of methods. Journal of Experimental Biology 211:2423–2430.

Avilés, J. M., B. G. Stokke, A. Moksnes, E. Røskaft, M. Asmul, and A. P. Møller. 2006. Rapid increase in cuckoo egg matching in a recently parasitized reed warbler population. Journal of Evolutionary Biology 19:1901–1910.

Baird, T., S. E. Solomon, and D. R. Tedstone. 1975. Localisation and characterisation of egg shell porphyrins in several avian species. British Poultry Science 16:201–208.

Barreira, A. S., D. A. Lijtmaer, S. C. Lougheed, and P. L. Tubaro. 2007. Subspecific and temporal variation in the structurally based coloration of the Ultramarine Grosbeak. Condor 109:187–192.

Bennett, A. T. D., I. C. Cuthill, and K. J. Norris. 1994. Sexual selection and the mismeasure of color. American Naturalist 144:848–860.

Bennett, A. T. D., and M. Théry. 2007. Avian color vision and coloration: multidisciplinary evolutionary biology. American Naturalist 169:S1–S6.

Berg, C. J., A. M. LaFountain, R. O. Prum, H. A. Frank, and M. J. Tauber. 2013. Vibrational and electronic spectroscopy of the retro-carotenoid rhodoxanthin in avian plumage, solid-state films, and solution. Archives of Biochemistry and Biophysics 539:142–155.

Björn, L. O., and H. Ghiradella. 2015. Spectral tuning in biology I: Pigments. Pp. 97–117 in L. O. Björn (editor), Photobiology, Springer, New York, NY.

Bridge, E. S., J. Hylton, M. D. Eaton, L. Gamble, and S. J. Schoech. 2007. Cryptic plumage signaling in *Aphelocoma* Scrub-Jays. Journal of Ornithology 149:123–130.

Brulez, K., P. Cassey, A. Meeson, I. Mikšík, S. L. Webber, A. G. Gosler, and S. J. Reynolds. 2014. Eggshell spot scoring methods cannot be used as a reliable proxy to determine pigment quantity. Journal of Avian Biology 45:94–102.

Brush, A. H. 1967. Pigmentation in the Scarlet Tanager, *Piranga olivacea*. Condor 69:549–559.

Brush, A. H. 1969. On the nature of "cotingin." Condor 71:431–433.

Brush, A. H. 1970. Pigments in hybrid, variant and melanic tanagers (birds). Comparative Biochemistry and Physiology 36:785–793.

Brush, A. H., and D. M. Power. 1976. House finch pigmentation: carotenoid metabolism and the effect of diet. Auk 93:725–739.

Brush, A. H., and H. Seifried. 1968. Pigmentation and feather structure in genetic variants of the Gouldian Finch, *Poephila gouldiae*. Auk 85:416–430.

Bulla, M., M. Šálek, and A. G. Gosler. 2012. Eggshell spotting does not predict male incubation but marks thinner areas of a shorebird's shells. Auk 129:26–35.

Burns, K. J., and A. J. Shultz. 2012. Widespread cryptic dichromatism and ultraviolet reflectance in the largest radiation of neotropical songbirds: implications of accounting for avian vision in the study of plumage evolution. Auk 129:211–221.

Cassey, P., M. E. Hauber, G. Maurer, and J. G. Ewen. 2012c. Sources of variation in reflectance spectrophotometric data: a quantitative analysis using avian eggshell colours. Methods in Ecology and Evolution 3:450–456.

Cassey, P., G. Maurer, C. Duval, J. G. Ewen, and M. E. Hauber. 2010b. Impact of time since collection on avian eggshell color: a comparison of museum and fresh egg specimens. Behavioral Ecology and Sociobiology 64:1711–1720.

Cassey, P., G. Maurer, P. G. Lovell, and D. Hanley. 2011. Conspicuous eggs and colourful hypotheses: testing the role of multiple influences on avian eggshell appearance. Avian Biology Research 4:185–195.

Cassey, P., I. Mikšík, S. J. Portugal, G. Maurer, J. G. Ewen, E. Zarate, M. A. Sewell, F. Karadas, T. Grim, and M. E. Hauber. 2012b. Avian eggshell pigments are not consistently correlated with colour measurements or egg constituents in two *Turdus* thrushes. Journal of Avian Biology 43:503–512.

Cassey, P., S. J. Portugal, G. Maurer, J. G. Ewen, R. L. Boulton, M. E. Hauber, and T. M. Blackburn. 2010a. Variability in avian eggshell colour: a comparative study of museum eggshells. PLoS One 5:e12054.

Cassey, P., G. H. Thomas, S. J. Portugal, G. Maurer, M. E. Hauber, T. Grim, P. G. Lovell, and I. Mikšík. 2012a. Why are birds' eggs colourful? Eggshell pigments co-vary with life-history and nesting ecology among British breeding non-passerine birds. Biological Journal of the Linnean Society 106:657–672.

Caves, E. M., M. Stevens, E. S. Iversen, and C. N. Spottiswoode. 2015. Hosts of avian brood parasites have evolved egg signatures with elevated information content. Proceedings of the Royal Society B 282:20150598.

Charter, M., Y. Leshem, I. Izhaki, and A. Roulin. 2014. Pheomelanin-based colouration is correlated with indices of flying strategies in the Barn Owl. Journal of Ornithology 156:309–312.

Cherry, M. I., and A. T. Bennett. 2001. Egg colour matching in an African cuckoo, as revealed by ultraviolet-visible reflectance spectrophotometry. Proceedings of the Royal Society B 268:565–571.

Cherry, M. I., and A. G. Gosler. 2010. Avian eggshell coloration: new perspectives on adaptive explanations. Biological Journal of the Linnean Society 100:753–762.

Chiao, C.-C., J. K. Wickiser, J. J. Allen, B. Genter, and R. T. Hanlon. 2011. Hyperspectral imaging of cuttlefish camouflage indicates good color match in the eyes of fish predators. Proceedings of the National Academy of Sciences USA 108:9148–9153.

Church, A. H. 1869. Researches on turacin, an animal pigment containing copper. Philosophical Transactions of the Royal Society of London 159:627–636.

Cuthill, I. 2006. Color perception. Pp. 3–40 *in* G. E. Hill and K. J. McGraw (editors), Bird coloration volume 1: mechanisms and measurements, Harvard University Press, Cambridge, MA.

Cuthill, I. C., A. T. D. Bennett, J. C. Partridge, and E. J. Maier. 1999. Plumage reflectance and the objective assessment of avian sexual dichromatism. American Naturalist 153:183–200.

Czeczuga, B. 1979. Carotenoids in the skin of certain species of birds. Comparative Biochemistry and Physiology B 62:107–109.

D'Alba, L., L. Kieffer, and M. D. Shawkey. 2012. Relative contributions of pigments and biophotonic nanostructures to natural color production: A case study in Budgerigar (*Melopsittacus undulatus*) feathers. Journal of Experimental Biology 215:1272–1277.

Davies, N. 2000. Cuckoos, Cowbirds and other cheats. T. & A. D. Poyser, London, UK.

Debecker, S., R. Sommaruga, T. Maes, R. Stoks, and G. Davidowitz. 2015. Larval UV exposure impairs adult immune function through a trade-off with larval investment in cuticular melanin. Functional Ecology 29:1292–1299.

Delhey, K., C. Burger, W. Fiedler, and A. Peters. 2010. Seasonal changes in colour: A comparison of structural, melanin- and carotenoid-based plumage colours. PLoS One 5:e11582.

Delhey, K., V. Delhey, B. Kempenaers, and A. Peters. 2014. A practical framework to analyze variation in animal colors using visual models. Behavioral Ecology 26:367–375.

Doucet, S. M., and G. E. Hill. 2009. Do museum specimens accurately represent wild birds? A case study of carotenoid, melanin, and structural colours in Long-tailed Manakins *Chiroxiphia linearis*. Journal of Avian Biology 40:146–156.

Doucet, S. M., D. J. Mennill, and G. E. Hill. 2007. The evolution of signal design in manakin plumage ornaments. American Naturalist 169:S62–S80.

Doucet, S. M., M. D. Shawkey, M. K. Rathburn, H. L. Mays, and R. Montgomerie. 2004. Concordant evolution of plumage colour, feather microstructure and a melanocortin receptor gene between mainland and island populations of a fairy-wren. Proceedings of the Royal Society B 271:1663–1670.

Dunn, P. O., J. K. Armenta, and L. A. Whittingham. 2015. Natural and sexual selection act on different axes of variation in avian plumage color. Science Advances 1:e1400155.

Dyck, J. 1966. Determination of plumage colours, feather pigments and structures by means of reflection spectrophotometry. Dansk Ornithologisk Forening 60:49–76.

Eaton, M. D. 2005. Human vision fails to distinguish widespread sexual dichromatism among sexually "monochromatic" birds. Proceedings of the National Academy of Sciences USA 102:10942–10946.

Eaton, M. D., and K. P. Johnson. 2007. Avian visual perspective on plumage coloration confirms rarity of sexually monochromatic North American passerines. Auk 124:155–161.

Eliason, C. M., R. Maia, and M. D. Shawkey. 2015. Modular color evolution facilitated by a complex nanostructure in birds. Evolution 69:357–367.

Endler, J. A. 1990. On the measurement and classification of colour in studies of animal colour patterns. Biological Journal of the Linnean Society 41:315–352.

Endler, J. A., and P. W. Mielke. 2005. Comparing entire colour patterns as birds see them. Biological Journal of the Linnean Society 86:405–431.

Endler, J. A., and M. Théry. 1996. Interacting effects of lek placement, display behavior, ambient light, and color patterns in three neotropical forest-dwelling birds. American Naturalist 148:421–452.

Endler, J. A., D. A. Westcott, J. R. Madden, and T. Robson. 2005. Animal visual systems and the evolution of color patterns: sensory processing illuminates signal evolution. Evolution 59:1795–1818.

Fitze, P. S., B. Tschirren, J. Gasparini, and H. Richner. 2007. Carotenoid-based plumage colors and immune function: is there a trade-off for rare carotenoids? American Naturalist 169:S137–S144.

Fox, D. L. 1962. Carotenoids of the Scarlet Ibis. Comparative Biochemistry and Physiology 5:31–43.

Fox, D. L., A. A. Wolfson, and J. W. McBeth. 1969. Metabolism of β-carotene in the American flamingo, *Phoenicopterus ruber*. Comparative Biochemistry and Physiology 29:1223–1229.

Friedman, N. R., C. M. Hofmann, B. Kondo, and K. E. Omland. 2009. Correlated evolution of migration and sexual dichromatism in the New World orioles (*Icterus*). Evolution 63:3269–3274.

Friedman, N. R., K. J. McGraw, and K. E. Omland. 2014a. History and mechanisms of carotenoid plumage evolution in the New World orioles (*Icterus*). Comparative Biochemistry and Physiology B 172-173:1–8.

Friedman, N. R., K. J. McGraw, and K. E. Omland. 2014b. Evolution of carotenoid pigmentation in caciques and meadowlarks (Icteridae): repeated gains of red plumage coloration by carotenoid C4-oxygenation. Evolution 68:791–801.

Galván, I., and A. Jorge. 2015. Dispersive Raman spectroscopy allows the identification and quantification of melanin types. Ecology and Evolution 5:1425–1431.

Galván, I., A. Jorge, K. Ito, K. Tabuchi, F. Solano, and K. Wakamatsu. 2013a. Raman spectroscopy as a non-invasive technique for the quantification of melanins in feathers and hairs. Pigment Cell Melanoma Research 26:917–923.

Galván, I., A. Jorge, F. Solano, and K. Wakamatsu. 2013b. Vibrational characterization of pheomelanin and trichochrome F by Raman spectroscopy. Spectrochimica Acta Part A, Molecular and Biomolecular Spectroscopy 110:55–59.

Galván, I., and A. P. Møller. 2013. Pheomelanin-based plumage coloration predicts survival rates in birds. Physiological and Biochemical Zoology 86:184–192.

Gamgee, A. 1895. On the relations of turacin and turacoporphyrin to the colouring matter of the blood. Proceedings of the Royal Society of London 59:339–342.

Gluckman, T.-L., and G. C. Cardoso. 2009. A method to quantify the regularity of barred plumage patterns. Behavioral Ecology and Sociobiology 63:1837–1844.

Goldsmith, T. H. 1980. Hummingbirds see near ultraviolet light. Science 207:786–788.

Goldsmith, T. H. 1990. Optimization, constraint, and history in the evolution of eyes. Quarterly Review of Biology 65:281–322.

Gomez, D., and M. Théry. 2007. Simultaneous crypsis and conspicuousness in color patterns: comparative analysis of a neotropical rainforest bird community. American Naturalist 169:S42–S61.

Gorchein, A., C. K. Lim, and P. Cassey. 2009. Extraction and analysis of colourful eggshell pigments using HPLC and HPLC/electrospray ionization tandem mass spectrometry. Biomedical Chromatography: BMC 23:602–606.

Gosler, A. G., O. R. Connor, and R. H. C. Bonser. 2011. Protoporphyrin and eggshell strength: preliminary findings from a passerine bird. Avian Biology Research 4:214–223.

Gosler, A. G., J. P. Higham, and S. James Reynolds. 2005. Why are birds' eggs speckled? Ecology Letters 8:1105–1113.

Götmark, F. 1987. White underparts in gulls function as hunting camouflage. Animal Behaviour 35:1786–1792.

Haase, E., S. Ito, A. Sell, and K. Wakamatsu. 1992. Melanin concentrations in feathers from wild and domestic pigeons. Journal of Heredity 83:64–67.

Hamilton, P. B. 1992. The use of high-performance liquid chromatography for studying pigmentation. Poultry Science 71:718–724.

Hanley, D., and S. M. Doucet. 2012. Does environmental contamination influence egg coloration? A long-term study in herring gulls. Journal of Applied Ecology 49:1055–1063.

Hanley, D., M. C. Stoddard, P. Cassey, and P. L. R. Brennan. 2013. Eggshell conspicuousness in ground nesting birds: do conspicuous eggshells signal nest location to conspecifics? Avian Biology Research 6:147–156.

Hart, N. S. 2001. Variations in cone photoreceptor abundance and the visual ecology of birds. Journal of Comparative Physiology A 187:685–697.

Hart, N. S., and D. M. Hunt. 2007. Avian visual pigments: characteristics, spectral tuning, and evolution. American Naturalist 169(Suppl. 1):S7–S26.

Hart, N. S., J. C. Partridge, I. C. Cuthill, and A. T. Bennett. 2000. Visual pigments, oil droplets, ocular media and cone photoreceptor distribution in two species of passerine bird: the Blue Tit (*Parus caeruleus* L.) and the Blackbird (*Turdus merula* L.). Journal of Comparative Physiology A 186:375–387.

Hart, N. S., and M. Vorobyev. 2005. Modelling oil droplet absorption spectra and spectral sensitivities of bird cone photoreceptors. Journal of Comparative Physiology A 191:381–392.

Håstad, O., and A. Odeen. 2008. Different ranking of avian colors predicted by modeling of retinal function in humans and birds. American Naturalist 171:831–838.

Hauber, M. E. 2014. The book of eggs: a lifesize guide to the eggs of six hundred of the world's bird species. University of Chicago Press, Chicago, IL.

Hausmann, F., K. E. Arnold, N. J. Marshall, and I. P. Owens. 2003. Ultraviolet signals in birds are special. Proceedings of the Royal Society B 270:61–67.

Hickey, J. J., and D. W. Anderson. 1968. Chlorinated hydrocarbons and eggshell changes in raptorial and fish-eating birds. Science 162:271–273.

Hill, G. E. 1991. Plumage coloration is a sexually selected indicator of male quality. Nature 350:337–339.

Hill, G. E., and K. J. McGraw. 2006a. Bird coloration volume 2: function and evolution. Harvard University Press, Cambridge, MA.

Hill, G. E., and K. J. McGraw. 2006b. Bird coloration volume 1: mechanisms and measurements. Harvard University Press, Cambridge, MA.

Hincke, M. T., Y. Nys, J. Gautron, K. Mann, A. B. Rodriguez-Navarro, and M. D. McKee. 2012. The eggshell: structure, composition and mineralization. Frontiers in Bioscience 17:1266–1280.

Hoi, H., and M. Griggio. 2008. Dual utility of a melanin-based ornament in Bearded Tits. Ethology 114:1094–1100.

Hudon, J. 1991. Unusual carotenoid use by the Western Tanager (*Piranga ludoviciana*) and its evolutionary implications. Canadian Journal of Zoology 69: 2311–2320.

Hudon, J., and A. H. Brush. 1992. Identification of carotenoid pigments in birds. Methods in Enzymology 213:312–321.

Hudon, J., A. P. Capparella, and A. H. Brush. 1989. Plumage pigment differences in manakins of the *Pipra erythrocephala* superspecies. Auk 106:34–41.

Hudon, J., and A. D. Muir. 1996. Characterization of the reflective materials and organelles in the bright irides of North American blackbirds (Icterinae). Pigment Cell Research 9:96–104.

Igic, B., K. Braganza, M. M. Hyland, H. Silyn-Roberts, P. Cassey, T. Grim, J. Rutila, C. Moskát, and M. E. Hauber. 2011. Alternative mechanisms of increased eggshell hardness of avian brood parasites relative to host species. Journal of The Royal Society Interface 8:1654–1664.

Igic, B., P. Cassey, T. Grim, D. R. Greenwood, C. Moskat, J. Rutila, and M. E. Hauber. 2012. A shared chemical basis of avian host-parasite egg colour mimicry. Proceedings of the Royal Society B 279:1068–76.

Igic, B., D. Fecheyr-Lippens, M. Xiao, A. Chan, D. Hanley, P. R. L. Brennan, T. Grim, G. I. N. Waterhouse, M. E. Hauber, and M. D. Shawkey. 2015. A nanostructural basis for gloss of avian eggshells. Journal of the Royal Society Interface 12:20141210.

Igic, B., D. R. Greenwood, D. J. Palmer, P. Cassey, B. J. Gill, T. Grim, P. L. R. Brennan, S. M. Bassett, P. F. Battley, and M. E. Hauber. 2009. Detecting pigments from colourful eggshells of extinct birds. Chemoecology 20:43–48.

Igic, B., M. E. Hauber, J. A. Galbraith, T. Grim, D. C. Dearborn, P. L. R. Brennan, C. Moskát, P. K. Choudhary, and P. Cassey. 2010. Comparison of micrometer- and scanning electron microscope-based measurements of avian eggshell thickness. Journal of Field Ornithology 81:402–410.

Ito, S., and K. Wakamatsu. 2003. Quantitative analysis of eumelanin and pheomelanin in humans, mice, and other animals: a comparative review. Pigment Cell Research 16:523–531.

Jacot, A., C. Romero-Diaz, B. Tschirren, H. Richner, and P. S. Fitze. 2010. Dissecting carotenoid from structural components of carotenoid-based coloration: a field experiment with Great Tits (*Parus major*). American Naturalist 176:55–62.

Jagannath, A., R. F. Shore, L. A. Walker, P. N. Ferns, and A. G. Gosler. 2007. Eggshell pigmentation indicates pesticide contamination. Journal of Applied Ecology 45:133–140.

Johnsen, A., S. Andersson, J. Ornborg, and J. T. Lifjeld. 1998. Ultraviolet plumage ornamentation affects social mate choice and sperm competition in Bluethroats (Aves: *Luscinia s. svecica*): a field experiment. Proceedings of the Royal Society B 265:1313–1318.

Johnson, N. K., J. V. Remsen, Jr., and C. Cicero. 1998. Refined colorimetry validates endangered subspecies of the Least Tern. Condor 100:18–26.

Karubian, J., W. R. Lindsay, H. Schwabl, and M. S. Webster. 2011. Bill coloration, a flexible signal in a tropical passerine bird, is regulated by social environment and androgens. Animal Behaviour 81:795–800.

Kelber, A., and D. Osorio. 2010. From spectral information to animal colour vision: experiments and concepts. Proceedings of the Royal Society B 277:1617–1625.

Kelber, A., M. Vorobyev, and D. Osorio. 2003. Animal colour vision—behavioural tests and physiological concepts. Biological Reviews 78:81–118.

Kemp, D. J., M. E. Herberstein, L. J. Fleishman, J. A. Endler, A. T. Bennett, A. G. Dyer, N. S. Hart, J. Marshall, and M. J. Whiting. 2015. An integrative framework for the appraisal of coloration in nature. American Naturalist 185:705–724.

Kennedy, G. Y., and H. G. Vevers. 1976. A survey of avian eggshell pigments. Comparative Biochemistry and Physiology B 55:117–123.

Kiff, L. F., and R. M. Zink. 2005. History, present status, and future prospects of avian eggshell collections in North America. Auk 122:994–999.

Kilner, R. M. 2006. The evolution of egg colour and patterning in birds. Biological Reviews of the Cambridge Philosophical Society 81:383–406.

Kim, M. H., T. A. Harvey, D. S. Kittle, H. Rushmeier, J. Dorsey, R. O. Prum, and D. J. Brady. 2012. 3D imaging spectroscopy for measuring hyperspectral patterns on solid objects. ACM Transactions on Graphics 31:1–11.

Kritzler, H. 1943. Carotenoids in the display and eclipse plumages of bishop birds. Physiological Zoology 16:241–255.

Krukenberg, C. F. W. 1882. Die federfarbstoffe der psittaciden. Vergleichend-physiologische Studien Reihe 2, Abtlg 2:29–36.

LaFountain, A. M., S. Kaligotla, S. Cawley, K. M. Riedl, S. J. Schwartz, H. A. Frank, and R. O. Prum. 2010. Novel methoxy-carotenoids from the burgundy-colored plumage of the Pompadour Cotinga *Xipholena punicea*. Archives of Biochemistry and Biophysics 504:142–153.

LaFountain, A. M., R. O. Prum, and H. A. Frank. 2015. Diversity, physiology, and evolution of avian plumage carotenoids and the role of carotenoid–protein interactions in plumage color appearance. Archives of Biochemistry and Biophysics 572:201–212.

Lahti, D. C. 2008. Population differentiation and rapid evolution of egg color in accordance with solar radiation. Auk 125:796–802.

Lee, P. L., and R. P. Prys-Jones. 2008. Extracting DNA from museum bird eggs, and whole genome amplification of archive DNA. Molecular Ecology Resources 8:551–560.

Li, Q., K.-Q. Gao, Q. Meng, J. A. Clarke, M. D. Shawkey, L. D'Alba, R. Pei, M. Ellison, M. A. Norell, and J. Vinther. 2012. Reconstruction of *Microraptor* and the evolution of iridescent plumage. Science 335:1215–1219.

Lim, C. K. 2004. High-performance liquid chromatography and mass spectrometry of porphyrins, chlorophylls and bilins. World Scientific Publishing Company, Hackensack, NJ.

Lovell, P. G., G. D. Ruxton, K. V. Langridge, and K. A. Spencer. 2013. Egg-laying substrate selection for optimal camouflage by quail. Current Biology 23:260–264.

Lowe, D. G. 1999. Object recognition from local scale-invariant features. Proceedings of the Seventh IEEE International Conference on Computer Vision, 1999.

Lowe, D. G. 2000. Towards a computational model for object recognition in IT cortex. Pp. 20–31 in S.-W. Lee, H. H. Bülthoff, and T. Poggio (editors), Biologically motivated computer vision: First IEEE International Workshop, BMCV 2000 Seoul, Korea, May 15–17, 2000 Proceedings, Springer, Berlin, Germany.

Lozano, G. A. 1994. Carotenoids, parasites, and sexual selection. Oikos 70:309–311.

Maan, M. E., and M. E. Cummings. 2012. Poison frog colors are honest signals of toxicity, particularly for bird predators. American Naturalist 179:E1–E14.

Mägi, M., R. Mänd, A. Konovalov, V. Tilgar, and S. J. Reynolds. 2012. Testing the structural–function hypothesis of eggshell maculation in the Great Tit: An experimental approach. Journal of Ornithology 153:645–652.

Maia, R., J. V. Caetano, S. N. Bao, and R. H. Macedo. 2009. Iridescent structural colour production in male blue-black grassquit feather barbules: the role of keratin and melanin. Journal of the Royal Society Interface 6:S203–S211.

Maia, R., C. M. Eliason, P.-P. Bitton, S. M. Doucet, M. D. Shawkey, and A. Tatem. 2013a. pavo: an R package for the analysis, visualization and organization of spectral data. Methods in Ecology and Evolution 4:906–913.

Maia, R., D. R. Rubenstein, and M. D. Shawkey. 2013b. Key ornamental innovations facilitate diversification in an avian radiation. Proceedings of the National Academy of Sciences USA 110:10687–10692.

Maley, J. M., and K. Winker. 2007. Use of juvenal plumage in diagnosing species limits: an example using buntings in the genus *Plectrophenax*. Auk 124:907–915.

Marchetti, K. 1993. Dark habitats and bright birds illustrate the role of the environment in species divergence. Nature 362:149–152.

Mason, N. A., A. J. Shultz, and K. J. Burns. 2014. Elaborate visual and acoustic signals evolve independently in a large, phenotypically diverse radiation of songbirds. Proceedings of the Royal Society B 281:20140967.

Maurer, G., S. J. Portugal, and P. Cassey. 2011. Review: an embryo's eye view of avian eggshell pigmentation. Journal of Avian Biology 42:494–504.

Maurer, G., S. J. Portugal, and P. Cassey. 2012. A comparison of indices and measured values of eggshell thickness of different shell regions using museum eggs of 230 European bird species. Ibis 154:714–724.

Maurer, G., S. J. Portugal, M. E. Hauber, I. Mikšík, D. G. D. Russell, P. Cassey, and B. Tschirren. 2015. First light for avian embryos: eggshell thickness and pigmentation mediate variation in development and UV exposure in wild bird eggs. Functional Ecology 29:209–218.

McGraw, K. J. 2004. European barn swallows use melanin pigments to color their feathers brown. Behavioral Ecology 15:889–891.

McGraw, K. J. 2006a. Mechanics of melanin coloration in birds. Pp. 243–294 *in* G. E. Hill and K. J. McGraw (editors), Bird coloration volume 1: mechanisms and measurements, Harvard University Press, Cambridge, MA.

McGraw, K. J. 2006b. Mechanics of carotenoid-based coloration. Pp. 177–242 *in* G. E. Hill and K. J. McGraw (editors), Bird coloration volume 1: mechanisms and measurements, Harvard University Press, Cambridge, MA.

McGraw, K. J. 2006c. Mechanics of uncommon colours: pterins, porphyrins, and psittacofulvins. Pp. 354–398 *in* G. E. Hill and K. J. McGraw (editors), Bird coloration volume 1: mechanisms and measurements, Harvard University Press, Cambridge, MA.

McGraw, K. J., and M. C. Nogare. 2005. Distribution of unique red feather pigments in parrots. Biology Letters 1:38–43.

McGraw, K. J., M. B. Toomey, P. M. Nolan, N. I. Morehouse, M. Massaro, and P. Jouventin. 2007. A description of unique fluorescent yellow pigments in penguin feathers. Pigment Cell Research 20:301–304.

McGraw, K. J., and K. Wakamatsu. 2004. Melanin basis of ornamental feather colors in male Zebra Finches. Condor 106:686–690.

McGraw, K. J., K. Wakamatsu, S. Ito, P. M. Nolan, P. Jouventin, F. S. Dobson, R. E. Austic, R. J. Safran, L. M. Siefferman, G. E. Hill, and R. S. Parker. 2004. You can't judge a pigment by its color: carotenoid and melanin content of yellow and brown feathers in swallows, bluebirds, penguins, and domestic chickens. Condor 106:390–395.

McKay, B. D. 2013. The use of digital photography in systematics. Biological Journal of the Linnean Society 110:1–13.

McKay, B. D., H. L. Mays, C.-T. Yao, D. Wan, H. Higuchi, and I. Nishiumi. 2014. Incorporating color into integrative taxonomy: analysis of the Varied Tit (*Sittiparus varius*) complex in East Asia. Systematic Biology 63:505–517.

McNett, G. D., and K. Marchetti. 2005. Ultraviolet degradation in carotenoid patches: live versus museum specimens of Wood Warblers (Parulidae). Auk 122:793–802.

Meadows, M. G., N. I. Morehouse, R. L. Rutowski, J. M. Douglas, and K. J. McGraw. 2011. Quantifying iridescent coloration in animals: a method for improving repeatability. Behavioral Ecology and Sociobiology 65:1317–1327.

Mendes-Pinto, M. M., A. M. LaFountain, M. C. Stoddard, R. O. Prum, H. A. Frank, and B. Robert. 2012. Variation in carotenoid-protein interaction in bird feathers produces novel plumage coloration. Journal of the Royal Society Interface 9:3338–3350.

Mikšík, I., V. Holáň, and Z. Deyl. 1996. Avian eggshell pigments and their variability. Comparative Biochemistry and Physiology B 113:607–612.

Møller, A. P. 1987. Variation in badge size in male House Sparrows *Passer domesticus*: evidence for status signalling. Animal Behaviour 35:1637–1644.

Montgomerie, R. 2006. Analyzing colors. Pp. 90–147 *in* G. E. Hill and K. J. McGraw (editors), Bird coloration volume 1: mechanisms and measurements, Harvard University Press, Cambridge, MA.

Montgomerie, R., B. Lyon, and K. Holder. 2001. Dirty ptarmigan: behavioral modification of conspicuous male plumage. Behavioral Ecology 12: 429–438.

Moreno, J., and J. L. Osorno. 2003. Avian egg colour and sexual selection: does eggshell pigmentation reflect female condition and genetic quality? Ecology Letters 6:803–806.

Murphy, T. G., M. F. Rosenthal, R. Montgomerie, and K. A. Tarvin. 2009. Female American goldfinches use carotenoid-based bill coloration to signal status. Behavioral Ecology 20:1348–1355.

Navarro, J. Y., and D. C. Lahti. 2014. Light dulls and darkens bird eggs. PLoS One 9:e116112.

Nicolaus, M., C. Le Bohec, P. Nolan, M. Gauthier-Clerc, Y. Le Maho, J. Komdeur, and P. Jouventin. 2007. Ornamental colors reveal age in the king penguin. Polar Biology 31:53–61.

Ödeen, A., and O. Håstad. 2013. The phylogenetic distribution of ultraviolet sensitivity in birds. BMC Evolutionary Biology 13:36.

Ödeen, A., S. Pruett-Jones, A. C. Driskell, J. K. Armenta, and O. Håstad. 2012. Multiple shifts between violet and ultraviolet vision in a family of passerine birds with associated changes in plumage coloration. Proceedings of the Royal Society B 279: 1269–1276.

Oliphant, L. W. 1987. Pteridines and purines as major pigments of the avian iris. Pigment Cell Research 1:129–131.

Oliphant, L. W. 1988. Cytology and pigments of non-melanophore chromatophores in the avian iris. Progress in Clinical and Biological Research 256:65–82.

Olson, V. A., and I. P. F. Owens. 1998. Costly sexual signals: are carotenoids rare, risky or required? Trends in Ecology and Evolution 13:510–514.

Oskam, C. L., J. Haile, E. McLay, P. Rigby, M. E. Allentoft, M. E. Olsen, C. Bengtsson, G. H. Miller, J. L. Schwenninger, C. Jacomb, R. Walter, A. Baynes, J. Dortch, M. Parker-Pearson, M. T. Gilbert, R. N. Holdaway, E. Willerslev, and M. Bunce. 2010. Fossil avian eggshell preserves ancient DNA. Proceedings of the Royal Society B 277:1991–2000.

Osorio, D., and A. D. Ham. 2002. Spectral reflectance and directional properties of structural coloration in bird plumage. Journal of Experimental Biology 205:2017–2027.

Osorio, D., and M. Vorobyev. 2005. Photoreceptor spectral sensitivities in terrestrial animals: adaptations for luminance and colour vision. Proceedings of the Royal Society B 272:1745–1752.

Pike, T. 2011. Using digital cameras to investigate animal colouration: estimating sensor sensitivity functions. Behavioral Ecology and Sociobiology 65:849–858.

Portugal, S. J., H. J. Cooper, C. G. Zampronio, L. L. Wallace, and P. Cassey. 2010a. Can museum egg specimens be used for proteomic analyses? Proteome Science 8:40.

Portugal, S. J., G. Maurer, and P. Cassey. 2010b. Eggshell permeability: a standard technique for determining interspecific rates of water vapor conductance. Physiological and Biochemical Zoology 83:1023–1031.

Prager, M., and S. Andersson. 2010. Convergent evolution of red carotenoid coloration in widowbirds and bishops (*Euplectes* spp.). Evolution 64:3609–3619.

Prum, R. O. 1999. The anatomy and physics of structural colours. Proceedings of the 22nd International Ornithological Congress, Durban, South Africa.

Prum, R. O. 2006. Anatomy, physics, and evolution of structural colors. Pp. 177–242 in G. E. Hill and K. J. McGraw (editors), Bird coloration volume 1: mechanisms and measurements, Harvard University Press, Cambridge, MA.

Prum, R. O., S. Andersson, and R. H. Torres. 2003. Coherent scattering of ultraviolet light by avian feather barbs. Auk 120:163–170.

Prum, R. O., A. M. LaFountain, C. J. Berg, M. J. Tauber, and H. A. Frank. 2014. Mechanism of carotenoid coloration in the brightly colored plumages of broadbills (Eurylaimidae). Journal of Comparative Physiology B 184:651–672.

Prum, R. O., and R. Torres. 2003a. Structural colouration of avian skin: convergent evolution of coherently scattering dermal collagen arrays. Journal of Experimental Biology 206:2409–2429.

Prum, R. O., and R. H. Torres. 2003b. A Fourier tool for the analysis of coherent light scattering by bio-optical nanostructures. Integrative and Comparative Biology 43:591–602.

Prum, R. O., R. H. Torres, S. Williamson, and J. Dyck. 1998. Coherent light scattering by blue feather barbs. Nature 396:28–29.

Pryke, S. R., and S. C. Griffith. 2006. Red dominates black: agonistic signalling among head morphs in the colour polymorphic Gouldian finch. Proceedings of the Royal Society B 273:949–957.

Renoult, J. P., A. Kelber, and H. M. Schaefer. 2015. Colour spaces in ecology and evolutionary biology. Biological Reviews 92:292–315.

Reynolds, S. J., G. R. Martin, and P. Cassey. 2009. Is sexual selection blurring the functional significance of eggshell coloration hypotheses? Animal Behaviour 78:209–215.

Rothstein, S. I. 1990. A model system for coevolution: avian brood parasitism. Annual Review of Ecology and Systematics 21:481–508.

Russell, B. J., and H. M. Dierssen. 2015. Use of hyperspectral imagery to assess cryptic color matching in *Sargassum* associated crabs. PLoS One 10:e0136260.

Russell, D. G. D., J. White, G. Maurer, and P. Cassey. 2010. Data-poor egg collections: tapping an important research resource. Journal of Afrotropical Zoology 6:77–82.

Ruuskanen, S., T. Laaksonen, J. Morales, J. Moreno, R. Mateo, E. Belskii, A. Bushuev, A. Järvinen, A. Kerimov, I. Krams, C. Morosinotto, R. Mänd, M. Orell, A. Qvarnström, F. Slater, V. Tilgar, M. E. Visser, W. Winkel, H. Zang, and T. Eeva. 2013. Large-scale geographical variation in eggshell metal and calcium content in a passerine bird (*Ficedula hypoleuca*). Environmental Science and Pollution Research 21:3304–3317.

Saks, L., I. Ots, and P. Horak. 2003. Carotenoid-based plumage coloration of male greenfinches reflects health and immunocompetence. Oecologia 134:301–307.

Santos, S. I. C. O., and J. T. Lumeij. 2007. Comparison of multiple-angle spectrometry of plumage versus individual feathers for the assessment of sexual dichromatism in the Long-tailed Finch (*Poephila acuticauda*). Journal of Ornithology 148:281–291.

Saranathan, V., J. D. Forster, H. Noh, S. F. Liew, S. G. Mochrie, H. Cao, E. R. Dufresne, and R. O. Prum. 2012. Structure and optical function of amorphous photonic nanostructures from avian feather barbs: a comparative small angle X-ray scattering (SAXS) analysis of 230 bird species. Journal of the Royal Society Interface 9:2563–2580.

Scharlemann, J. P. W. 2001. Museum egg collections as stores of long-term phenological data. International Journal of Biometeorology 45:208–211.

Schmitz-Ornés, A. 2006. Using colour spectral data in studies of geographic variation and taxonomy of birds: examples with two hummingbird genera, *Anthracothorax* and *Eulampis*. Journal of Ornithology 147:495–503.

Seddon, N., J. A. Tobias, M. Eaton, A. Ödeen, and B. E. Byers. 2010. Human vision can provide a valid proxy for avian perception of sexual dichromatism. Auk 127:283–292.

Shawkey, M. D., S. L. Balenger, G. E. Hill, L. S. Johnson, A. J. Keyser, and L. Siefferman. 2006a. Mechanisms of evolutionary change in structural plumage coloration among bluebirds (*Sialia* spp.). Journal of the Royal Society Interface 3:527–532.

Shawkey, M. D., A. M. Estes, L. Siefferman, and G. E. Hill. 2005. The anatomical basis of sexual dichromatism in non-iridescent ultraviolet-blue structural coloration of feathers. Biological Journal of the Linnean Society 84:259–271.

Shawkey, M. D., M. E. Hauber, L. K. Estep, and G. E. Hill. 2006b. Evolutionary transitions and mechanisms of matte and iridescent plumage coloration in grackles and allies (Icteridae). Journal of the Royal Society Interface 3:777–786.

Shawkey, M. D., and G. E. Hill. 2005. Carotenoids need structural colours to shine. Biology Letters 1:121–124.

Shawkey, M. D., and G. E. Hill. 2006. Significance of a basal melanin layer to production of non-iridescent structural plumage color: evidence from an amelanotic Steller's Jay (*Cyanocitta stelleri*). Journal of Experimental Biology 209:1245–1250.

Shawkey, M. D., V. Saranathan, H. Palsdottir, J. Crum, M. H. Ellisman, M. Auer, and R. O. Prum. 2009. Electron tomography, three-dimensional Fourier analysis and colour prediction of a three-dimensional amorphous biophotonic nanostructure. Journal of the Royal Society Interface 6:S213–S220.

Shultz, A. J., and K. J. Burns. 2013. Plumage evolution in relation to light environment in a novel clade of neotropical tanagers. Molecular Phylogenetics and Evolution 66:112–125.

Smith, E., and G. Dent. 2005. Modern Raman spectroscopy: a practical approach. Wiley, New York, NY.

Smith, I. D., and H. S. Perdue. 1966. Isolation and tentative identification of the carotenoids present in chicken skin and egg yolks. Poultry Science 45:577–581.

Smith, T. B., P. P. Marra, M. S. Webster, I. Lovette, H. L. Gibbs, R. T. Holmes, K. A. Hobson, S. Rohwer, and R. Prum. 2003. A call for feather sampling. Auk 120:218–221.

Solomon, S. 1987. Egg shell pigmentation. Pp. 147–158 *in* R. G. Wells and C. G. Belyavin (editors), Egg quality: current problems and recent advances, Butterworths, London, UK.

Sparks, N. H. C. 2011. Eggshell pigments—from formation to deposition. Avian Biology Research 4:162–167.

Spottiswoode, C. N., and M. Stevens. 2010. Visual modeling shows that avian host parents use multiple visual cues in rejecting parasitic eggs. Proceedings of the National Academy of Sciences USA 107: 8672–8676.

Starling, M., R. Heinsohn, A. Cockburn, and N. E. Langmore. 2006. Cryptic gentes revealed in pallid cuckoos *Cuculus pallidus* using reflectance spectrophotometry. Proceedings of the Royal Society B 273:1929–1934.

Stevens, M. 2011. Avian vision and egg colouration: concepts and measurements. Avian Biology Research 4:168–184.

Stevens, M., C. A. Párraga, I. C. Cuthill, J. C. Partridge, and T. S. Troscianko. 2007. Using digital photography to study animal coloration. Biological Journal of the Linnean Society 90:211–237.

Stevens, M., M. C. Stoddard, and J. P. Higham. 2009. Studying primate color: towards visual system-dependent methods. International Journal of Primatology 30:893–917.

Stoddard, M. C., R. M. Kilner, and C. Town. 2014. Pattern recognition algorithm reveals how birds evolve individual egg pattern signatures. Nature Communications 5:4117.

Stoddard, M. C., K. L. A. Marshall, and R. M. Kilner. 2011. Imperfectly camouflaged avian eggs: artefact or adaptation? Avian Biology Research 4:196–213.

Stoddard, M. C., and R. O. Prum. 2008. Evolution of avian plumage color in a tetrahedral color space: a phylogenetic analysis of New World buntings. American Naturalist 171:755–776.

Stoddard, M. C., and R. O. Prum. 2011. How colorful are birds? Evolution of the avian plumage color gamut. Behavioral Ecology 22:1042–1052.

Stoddard, M. C., and M. Stevens. 2010. Pattern mimicry of host eggs by the common cuckoo, as seen through a bird's eye. Proceedings of the Royal Society B 277:1387–1393.

Stoddard, M. C., and M. Stevens. 2011. Avian vision and the evolution of egg color mimicry in the common cuckoo. Evolution 65:2004–2013.

Stradi, R., G. Celentano, M. Boles, and F. Mercato. 1997. Carotenoids in bird plumage: the pattern in a series of red-pigmented Carduelinae. Comparative Biochemistry and Physiology B 117:85–91.

Stradi, R., G. Celentano, and D. Nava. 1995a. Separation and identification of carotenoids in bird's plumage by high-performance liquid chromatography-diode-array detection. Journal of Chromatography B: Biomedical Sciences and Applications 670:337–348.

Stradi, R., G. Celentano, E. Rossi, G. Rovati, and M. Pastore. 1995b. Carotenoids in bird plumage—I. the carotenoid pattern in a series of Palearctic Carduelinae. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 110:131–143.

Stradi, R., J. Hudon, G. Celentano, and E. Pini. 1998. Carotenoids in bird plumage: the complement of yellow and red pigments in true woodpeckers (Picinae). Comparative Biochemistry and Physiology B 120:223–230.

Stradi, R., E. Pini, and G. Celentano. 2001. The chemical structure of the pigments in *Ara macao* plumage. Comparative Biochemistry and Physiology B 130:57–63.

Stradi, R., E. Rossi, G. Celentano, and B. Bellardi. 1996. Carotenoids in bird plumage: the pattern in three *Loxia* species and in *Pinicola enucleator*. Comparative Biochemistry and Physiology B 113:427–432.

Strain, H. H. 1934. Carotene VIII. separation of carotenes by adsorption. Journal of Biological Chemistry 105:523–535.

Test, F. H. 1969. Relation of wing and tail color of the woodpeckers *Colaptes auratus* and *C. cafer* to their food. Condor 71:206–211.

Thomas, D. B., M. E. Hauber, D. Hanley, G. I. N. Waterhouse, S. Fraser, and K. C. Gordon. 2015. Analysing avian eggshell pigments with Raman spectroscopy. Journal of Experimental Biology 218:2670–2674.

Thomas, D. B., C. M. McGoverin, K. J. McGraw, H. F. James, and O. Madden. 2013. Vibrational spectroscopic analyses of unique yellow feather pigments (spheniscins) in penguins. Journal of the Royal Society Interface 10:20121065.

Thomas, D. B., K. J. McGraw, M. W. Butler, M. T. Carrano, O. Madden, and H. F. James. 2014a. Ancient origins and multiple appearances of carotenoid-pigmented feathers in birds. Proceedings of the Royal Society B 281:20140806.

Thomas, D. B., K. J. McGraw, H. F. James, and O. Madden. 2014b. Non-destructive descriptions of carotenoids in feathers using Raman spectroscopy. Analytical Methods 6:1301–1308.

Thomas, D. B., P. C. Nascimbene, C. J. Dove, D. A. Grimaldi, and H. F. James. 2014c. Seeking carotenoid pigments in amber-preserved fossil feathers. Scientific Reports 4:5226.

Troscianko, J., and M. Stevens. 2015. Image calibration and analysis toolbox—a free software suite for objectively measuring reflectance, colour and pattern. Methods in Ecology and Evolution 6:1320–1331.

Troy, D. M., and A. H. Brush. 1983. Pigments and feather structure of the Redpolls, *Carduelis flammea* and *C. hornemanni*. Condor 85:443–446.

Tswett, M. 1906. Physikalisch-chemische studien über das chlorophyll. Die adsorption. Berichte der Deutschen botanischen Gesellschaft 24:316–326.

Tubaro, P. L., D. A. Lijtmaer, and S. C. Lougheed. 2005. Cryptic dichromatism and seasonal color variation in the Diademed Tanager. Condor 107:648–656.

Underwood, T. J., and S. G. Sealy. 2002. Adaptive significance of egg coloration. Pp. 280–298 *in* D. C. Deeming (editor), Avian incubation, behaviour, environment and evolution, Oxford University Press, Oxford, UK.

Veronelli, M., G. Zerbi, and R. Stradi. 1995. In situ resonance Raman spectra of carotenoids in bird's feathers. Journal of Raman Spectroscopy 26:683–692.

Vinther, J. 2015. A guide to the field of palaeo colour—melanin and other pigments can fossilise: reconstructing colour patterns from ancient organisms can give new insights to ecology and behaviour. BioEssays 37:643–656.

Vinther, J., D. E. Briggs, J. Clarke, G. Mayr, and R. O. Prum. 2010. Structural coloration in a fossil feather. Biology Letters 6:128–131.

Vinther, J., D. E. Briggs, R. O. Prum, and V. Saranathan. 2008. The colour of fossil feathers. Biology Letters 4:522–525.

Völker, O. 1934. Die abhangigkeit der lipochrombildung bie vogeln von pflanzlichen carotinoiden. Journal für Ornithologie 82:439–450.

Völker, O. 1938. Porphyrin in vogelfedern. Journal für Ornithologie 86:436–456.

Vorobyev, M. 2003. Coloured oil droplets enhance colour discrimination. Proceedings of the Royal Society B 270:1255–1261.

Vorobyev, M., R. Brandt, D. Peitsch, S. B. Laughlin, and R. Menzel. 2001. Colour thresholds and receptor noise: behaviour and physiology compared. Vision Research 41:639–653.

Vorobyev, M., and D. Osorio. 1998. Receptor noise as a determinant of colour thresholds. Proceedings of the Royal Society B 265:351–358.

Vorobyev, M., D. Osorio, A. T. Bennett, N. J. Marshall, and I. C. Cuthill. 1998. Tetrachromacy, oil droplets and bird plumage colours. Journal of Comparative Physiology A 183:621–633.

Vukusic, P., and D. G. Stavenga. 2009. Physical methods for investigating structural colours in biological systems. Journal of the Royal Society Interface 6:S133–S148.

Wackenroder, H. 1831. Ueber das oleum radicis dauci aetherum, das carotin, den carotenzucker und den officinellen succus cauci; so wie auch über das mannit, welches in dem möhrensafte durch eine besondere art der gährung gebildet wird. Geigers Magazin der Pharmazie 33:144–172.

White, T. E., R. L. Dalrymple, D. W. A. Noble, J. C. O'Hanlon, D. B. Zurek, and K. D. L. Umbers. 2015. Reproducible research in the study of biological coloration. Animal Behaviour 106:51–57.

With, T. K. 1978. On porphyrins in feathers of owls and bustards. International Journal of Biochemistry 9:893–895.

Woodward, L. A. 1967. General Introduction. Pp. 1–6 *in* H. A. Szymanski (editor), Raman spectroscopy: theory and practice, Plenum Press, New York, NY.

Yang, C., Y. Cai, and W. Liang. 2013. Eggs mimicry of Common Cuckoo (*Cuculus canorus*) utilizing Ashy-throated Parrotbill (*Paradoxornis alphonsianus*) host. Chinese Birds 4:51–56.

Zhou, J., J. Shang, F. Ping, and G. Zhao. 2012. Alcohol extract from *Vernonia anthelmintica* (L.) wild seed enhances melanin synthesis through activation of the p38 MAPK signaling pathway in B16F10 cells and primary melanocytes. Journal of Ethnopharmacology 143:639–647.