

CHAPTER TWO

Getting under the Skin

A CALL FOR SPECIMEN-BASED RESEARCH ON THE INTERNAL ANATOMY OF BIRDS*

Helen F. James

Abstract. Study of the comparative internal anatomy of birds is undergoing a renaissance, spurred by technological and methodological advances. Our ability to image the soft anatomy and bones in 3D using x-ray computed tomography (CT), magnetic resonance imaging (MRI), and optical surface imaging has opened the door to a wide range of analyses using avian skeletal and anatomical specimens. For anatomical specimens, simple staining techniques that enhance the contrast between different soft tissues, and at the same time raise the opacity of soft tissues to x-rays, enable the simultaneous 3D visualization of skeletal and soft tissue anatomy. Image processing software further allows anatomical features such as individual muscles to be segregated and measured on a computer monitor, without necessitating dissection of the anatomical specimen. Perfusion techniques can allow the vascular or respiratory system to be similarly imaged. For the skeleton, CT and optical

scans enable the production of detailed computer models of the bones for biometric and biomechanical studies. Online repositories of morphological image files can make internal anatomy widely accessible. Avian skeletal and anatomical collections are far less comprehensive than traditional study skin collections, yet they represent a wealth of relatively unexplored phenotypic variation in birds. The purpose of this chapter is to review and encourage the use of these techniques in the study of avian phenotypes, emphasizing the various specimen types that can be used as well as the deeper understanding of the ecological and behavioral context of the phenotype that emerges from such studies.

Key Words: anatomical specimens, avian paleontology, computed tomography, functional anatomy, geometric morphometrics, skeletons, spirit collections, 3D imaging.

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Advances in instrumentation and methods for studying vertebrate morphology offer exciting new ways to reveal and analyze internal phenotypic variation in birds. Emblematic of these advances are the captivating 3D visualizations of internal anatomy that have recently appeared in a diversity of journals (e.g., Figure 2.1). The primary tools employed to create those images are computed tomography (CT), magnetic resonance imaging (MRI), and laser or other optical surface scanning technologies, combined with software programs for creating 3D images from the scans (Chatham and Blackband 2001, Rosset et al. 2004, Goldman 2007, Marshall and Stutz 2012). These approaches, together with a suite of other techniques, some old and some new, have opened a frontier in our ability to see and study the insides of birds.

My objective in this chapter is to spur ornithologists to adopt these techniques, to incorporate internal anatomical traits more frequently in their study designs, and to collect the anatomical specimens that make these studies possible. The chapter touches on topics in avian biology to which studies of skeletal and anatomical specimens principally contribute, and highlights emerging techniques in imaging, data gathering, and data analysis that can facilitate research using those collections. I also offer brief comments on the readiness of avian skeletal and anatomical collections to fulfill this research mission.

In consonance with the theme of this volume, I omit discussion of several important research areas that admittedly make good use of skeletal and anatomical collections, but that do not aim specifically to understand the phenotype in an evolutionary or ecological context. Thus, I discuss avian paleontology but not zooarchaeology, and I omit ancient biomolecules such as the gene fragments and proteins that are often preserved in skeletal and sometimes anatomical specimens. Good reviews are available on these topics, such as Wiley et al. (Chapter 6, this volume) for stable isotope analysis of avian museum specimens, Wood and De Pietri (2015) for emerging paleo-ornithological techniques including the study of ancient biomolecules, and McCormack et al. (Chapter 9, this volume) for genomic approaches that utilize specimens.

AVIAN ANATOMICAL AND SKELETAL COLLECTIONS

Traditional museum study skin collections lie at the heart of our knowledge about the species and taxonomy of birds, their geographic distributions, plumages, and other external traits. The major scientific collections of birds were built up primarily from the 1880s through the 1960s (Winker 1996, Livezey 2003), a period when much ornithological research effort was devoted to establishing basic information about the systematics and biogeography of the world's birds. The avian study skin was

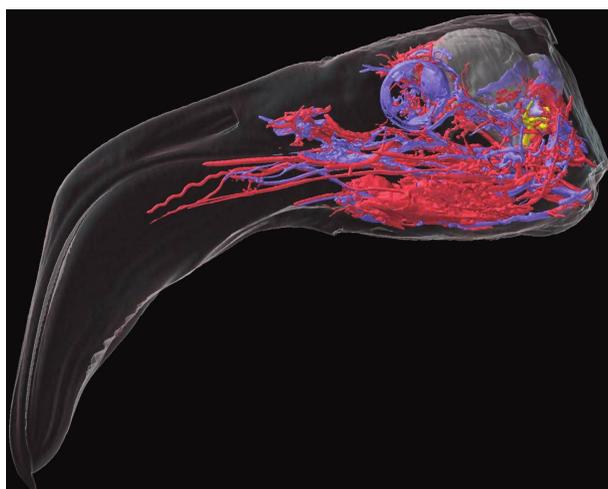


Figure 2.1. Visualization of the cranial vasculature of an American Flamingo (*Phoenicopterus ruber*), created by injecting a latex/barium medium into the vasculature and producing a CT scan. (From Holliday et al. 2006.)

adopted early on as a common unit of comparison for this research, a unit that was also conveniently fungible for curators engaged in trades with other institutions. When, in the 1960s and 1970s, the attention of ornithologists turned increasingly to ecology, the study skin was still the specimen of choice because it preserved external traits of birds that could be compared with those of birds studied in the field.

A consequence of this love for the beautiful study skin is that collectors of birds have preserved far fewer skeletal and anatomical specimens than skins. As an example, at the National Museum of Natural History (NMNH; Washington, DC), the avian holdings fall out as roughly 84% study skins representing 75% of the world's species, but only 11% skeletons representing 54% of the species and 5% anatomical specimens representing 44% of the species; eggs and nests are excluded from these calculations. The skeletal and anatomical holdings at NMNH are the most comprehensive anywhere, yet they continue to lag far behind the study skin collection, even though, in recent decades, the curators at NMNH have placed a priority on closing those gaps (Figure 2.2). The emphasis on study skins for alpha taxonomy is further highlighted by the composition of avian type collections. Again using the NMNH as an example, 3,971 type specimens are in the bird collection, only five of

which are anything other than a study skin. It almost goes without saying that the phenotypic variation that lies beneath the skin of birds has not been studied to nearly the same extent as has the variation observable in study skins, particularly when it comes to intrageneric and intraspecific patterns. Thus, specimens that capture the internal anatomy of birds can be considered as part of the “extended specimen” that is the focus of this book (see Chapter 1, this volume).

COMPARATIVE ANATOMY

The comparative study of avian anatomy, traditionally based on dissection and histology, has fallen into a relatively quiet period in recent decades (Livezey 2003), although certainly some notable work has continued to appear (e.g., Moreno and Carrascal 1993, Patak and Baldwin 1998, Maxwell and Larsson 2007). The rise in interest in avian ecology and behavior, and the supplanting of comparative anatomy with molecular genetics in the field of systematics, must partly account for this. The result has been that fluid-preserved avian specimens have by and large been languishing in their jars, and curators have received very few requests for their use. Yet this situation is beginning to reverse itself, and anatomical specimens are coming back in demand thanks to exciting

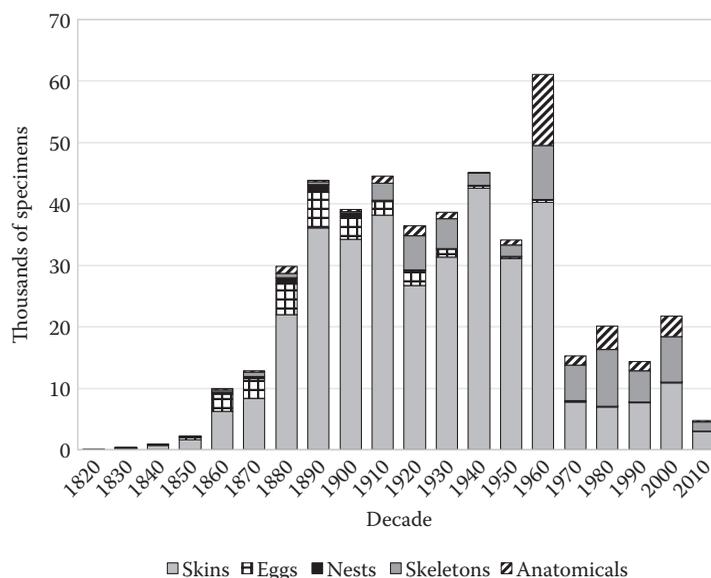


Figure 2.2. Bird specimens added to the scientific collection of the National Museum of Natural History, Smithsonian Institution, by decade and specimen type. The 2010 time bin represents the first half of the decade.

developments in imaging and computer-based analytical methods.

Some recent examples of this trend include studies of the vascular system of the head of flamingoes that revealed a previously unknown paralingual sinus that may be part of the filter feeding mechanism (Holliday et al. 2006), a study of the anatomy of the knee joint of ostriches that detailed the skeletomuscular system associated with their unusual second patella (Chadwick et al. 2014), and a study of the tongue apparatus of waterfowl that characterized anatomical differences between grazers and filter-feeders and used them to interpret key fossil anatids (Li and Clarke 2015). Each of these studies used CT scanning to create 3D anatomical models. In the flamingo study, a barium/latex medium was injected into the carotid artery and jugular vein to produce spectacular scans of the vascular system (Figure 2.1). In the ostrich study, MRI was used to visualize the muscular system and CT to image the bones of the knee joint. Finally, the study of the tongue apparatus in waterfowl imaged both bones and muscles simultaneously, using CT scanning enhanced with iodine-based perfusion staining of the whole specimen. It was then possible to segment the individual muscles using computer models and create a 3D, in situ, virtual dissection (Figure 2.3). This technique is part of a revolution sparked by Metscher (2009), who highlighted the advantages of staining whole

fluid-preserved anatomical specimens of small animals with iodine or iodine potassium iodide (I_2KI) to enhance the contrast between soft tissues, such as individual muscles and organs, in CT scans. A mineral stain like iodine also raises the opacity of soft tissues to x-rays, making them similar enough to bone in opacity that bones and soft anatomy can be visualized in a single CT scan. This simple but exciting insight about the advantages of iodine-based staining facilitates simultaneous visualization and precise measurements of the soft anatomy and skeleton in 3D. It opens the door to detailed anatomical and biomechanical investigations of fluid-preserved specimens, with traditional dissection and histology needed only to confirm the computer-based interpretations. Further experiments on iodine-based staining of anatomical specimens for CT scanning have refined the methods and adapted them for use with larger animals (e.g., Gignac and Kley 2014, Li et al. 2015).

SYSTEMATICS OF EXTANT SPECIES

Skeletons and anatomical specimens have long played a strong role in avian systematics. In classic taxonomies, higher categories of birds were arranged in part based on a small number of important anatomical traits, drawn, for example, from the hind-limb myology and configuration of palatal bones (e.g., Fürbringer 1888, Gadow 1892).

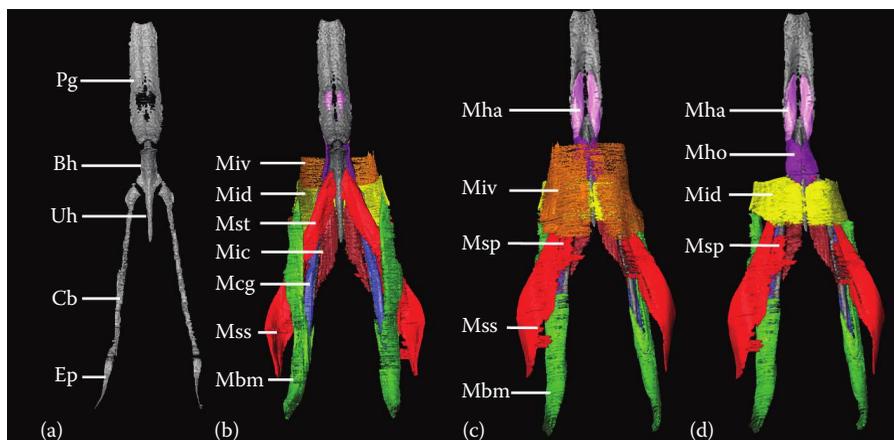


Figure 2.3. Virtual dissection of the tongue apparatus in a Canada Goose (*Branta canadensis*), created by segmenting individual muscles in enhanced-contrast CT scans. Dorsal (a, b) and ventral (c, d) views. Abbreviations: Bh, basihyal; Cb, ceratobranchial; Ep, epibranchial; Pg, paraglossal; Uh, urohyal; Mbm, M. branchiomandibularis; Mcg, M. ceratoglossus; Mha, M. hyoglossus anterior; Mho, M. hyoglossus obliquus; Mic, M. interceratobranchialis; Mid, M. intermandibularis dorsalis; Miv, M. intermandibularis ventralis; Msp, M. serphyoideus; Mss, M. stylohyoideus; Mst, M. stylohyoideus. (From Li and Clarke 2015.)

Other authors took a fine-scale approach, using comparative myology and osteology to inform the classification of all taxa within a group or clade, such as in Raikow's (1993) study of woodcreepers and Bertelli and Giannini's (2005) study of penguins. Despite this deep history of anatomical investigation, the internal anatomy of many groups of birds, for example, in the Passeriformes, has yet to receive very much attention at all. As one example, the notarium—a series of fused thoracic vertebrae independent of the synsacrum—was thought to be absent in Passeriformes until a relatively recent osteological survey revealed these structures to be widespread in the order, including in such familiar taxa as the European Starling (*Sturnus vulgaris*) and the crossbills (*Loxia* spp.; Storer 1982, James 2009). Establishing the systematic distribution of a trait that has evolved repeatedly, like the notarium, can open the door to studies of the trait's function and evolutionary development.

Beginning in the 1970s for anatomical specimens (e.g., Raikow 1977) and somewhat later for skeletons (e.g., Livezey 1986), internal anatomy has been used to build character state matrices for phylogenetic analysis, primarily using the parsimony criterion. The ambitious morphological analysis of higher-level classification of birds by Livezey and Zusi (2006, 2007) is a prime example. However, by now, molecular systematics has understandably supplanted morphology as the main source of data for phylogenetics of modern birds (see Jarvis et al. 2014, Prum et al. 2015). Yet anatomical characters remain relevant in systematics because they are a valuable data partition often used in tandem with molecular data (Fleischer et al. 2001, James et al. 2003), because they enable interpretation of the fossil record (discussed later), and because they reveal evolutionary patterns in the phenotype.

MORPHOLOGY AND BEHAVIOR

Research that extends beyond the boundaries of traditional systematics to place morphology in an ecological or behavioral context can be particularly satisfying because it provides a more holistic explanation of morphological diversity. For example, Bostwick et al. (2012) used CT scanning to document the unique construction of forelimb bones in the Club-winged Manakin (*Machaeropterus deliciosus*), and then related the bony morphology to the evolution of unusual feathers via sexual

selection (see Chapter 5, this volume). Riede et al. (2015) examined the myology and histology of the syrinx in male Pectoral Sandpipers (*Calidris melanotos*), detailing the anatomical specializations in the inflatable esophagus that allow them to produce hooting calls during courtship display. Prince et al. (2011) showed that sexual dimorphism in morphology of the syrinx and upper vocal tract underlies the larger vocal repertoires and more frequent vocalizations of male versus female European Starlings. As a final example, Krilow and Iwaniuk (2015) identified seasonal changes in the volumes of two specific brain regions, the striatopallidal complex and acropallium, that occur in association with drumming behavior in male Ruffed Grouse (*Bonasa umbellus*).

PALEONTOLOGY

The portion of an avian phenotype that is most likely to fossilize is the skeleton, which, as a consequence, has not experienced the same slow rate of use in research as have anatomical specimens. Indeed, avian paleontology—including considerable work with skeleton specimens—has a burgeoning literature that is bringing forth new findings for every major geological period in the history of birds. Some recent highlights include a greatly improved and still expanding Mesozoic fossil record of birds (e.g., Carvalho et al. 2015, Hu et al. 2015), Paleogene fossils from several continents that are particularly informative about the biogeographic and temporal origins of modern bird orders (Mayr 2009), a diverse Neogene record that encompasses extinct avian giants as well as fossils that are clearly relevant to modern genera and species (e.g., Olson and Rasmussen 2001, Tambussi et al. 2012), and rich Quaternary island records that are informative about the history of interactions between birds and people (e.g., James 1995, Worthy and Holdaway 2002, Steadman 2006, Meijer et al. 2013).

Phylogenetic placement of fossils relies on comparative osteology, and consequently many osteological character state matrices have been developed primarily to analyze fossils (e.g., Mayr and Clarke 2003, James 2004, Worthy 2009, Smith 2010). There is also great interest in how best to make use of the fossil record to reliably calibrate molecular phylogenetic trees to a timescale (e.g., Ksepka and Clarke 2015, Smith and Ksepka 2015). Figure 2.4 shows the type specimen of *Limnofregata azygosternon*,



Figure 2.4. Holotype of *Limnofregata azygosternon*, USNM 22753, an Eocene relative of frigatebirds from Wyoming that constrains the minimum age of the split between *Fregatidae* and *Suloidea* (including *Sulidae*, *Phalacrocoracidae*, and *Anhingidae*; see Smith and Ksepka 2015).

a fossil relative of frigatebirds that is a minimum of 51.8 million years old, and illustrates the quality of preservation in older fossils that is particularly helpful for calibrating the molecular phylogeny of modern birds.

Once the systematic position and time frame of a fossil have been worked out, its morphology can be further studied to answer diverse questions about the history of avian phenotypes. CT and optical scanning are excellent tools to visualize and model fossils, and are good companions to more traditional techniques like caliper morphometrics and histology. To give just one of many possible examples, the ability to create virtual endocasts of the braincase from CT scans has facilitated comparisons of fossil and modern neuroanatomy (Ksepka et al. 2012, Smith and Clarke 2012, Tambussi et al. 2015).

MORPHOMETRICS

Morphometrics, or the measurement and analysis of linear dimensions, shapes, and volumes, is employed in research on all topics surrounding the internal anatomy of birds. For instance, caliper measurements of the skeleton have long been used to characterize the diversity of avian phenotypes in relation to ecology and evolutionary diversity. A classic example is Hertel's (1994) discriminant function analysis of cranial morphometrics in vultures, in relationship to feeding

guilds. If the same study were performed today, we could characterize skull geometry using 3D landmark-based geometric morphometrics, as Kulemeyer et al. (2009) did in their analysis of morphological integration of the neurocranium and bony beak of corvids. Kulemeyer et al.'s study generated several hypotheses about the adaptation of beak shapes and binocular visual fields in corvids, as they relate to pecking compared to probing foraging modes, and raised a possible relationship among skull shape, head posture, and their propensity to take lengthy flights (as indicated by wing tip shape; see Chapter 8, this volume). This is just one example of how the ability to take precise measurements, including geometric measurements based on landmarks and measurements of surface areas and volumes, is leading to a deeper understanding of avian morphology and stimulating new hypotheses to explain the ecological, functional, and developmental context of morphologies.

Geometric morphometric analysis is an umbrella term that encompasses a variety of specific analytical approaches based on landmarks, but it has some general limitations when it comes to birds. Because birds lack teeth and have many fused bones with few open sutures in adulthood, they provide fewer good skeletal landmarks than is typical of other vertebrate groups. It may thus be necessary to resort to interpolated or "semi-landmarks" in birds, as Kulemeyer et al. (2009) did to characterize skull

and bill shape. To examine whether pygostyle shape is related to locomotory mode in waterbirds, Felice and O'Connor (2014) compared two-dimensional outlines of the bones using elliptical Fourier analysis, another variant of geometric morphometrics. As a final example, Sievwright and MacLeod (2012) used an eigensurface analysis of the proximal humerus of diurnal raptors (Figure 2.5) to show that the fine-scale geometry of just one end of an important bone can be correlated with flight style and migratory behavior.

Another limitation of geometric morphometric approaches is that they are most appropriate for single bony elements or articulated structures like the skull, and less applicable when the study concerns multiple bony elements that have no fixed geometric relationship to each other, such as the bones in a limb. This helps to explain why basic caliper morphometrics are still in widespread use. Recent examples include a regression analysis of scaling relationships between skeletal measurements and avian body mass (Field et al. 2013), and

a study showing that skeletal limb proportions in bluebirds (*Sialia* spp.) are convergent on those of flycatchers (Corbin et al. 2013).

In the context of morphometrics, I would emphasize that most studies in the past have examined interspecific patterns. With notable exceptions (e.g., McKittrick 1986, Johnston 1990, Rising 2001, Murphy 2007, Krilow and Iwaniuk 2015), intraspecific variation is an aspect of avian biology that has barely been touched upon using internal phenotypic characters. Instead, the common way to characterize intraspecific variation using museum specimens is through analysis of external measurements and plumages of study skins. Arguably, the skeleton offers more and perhaps better landmarks, in the sense that skeletal measurements are likely to have higher repeatability and less variability within an individual. For example, studying skeletal museum specimens can avoid seasonal changes due to the growth and wear of feathers and rhamphotheca or to body mass changes that might add unwanted variance if that is not the topic of study. In addition, new

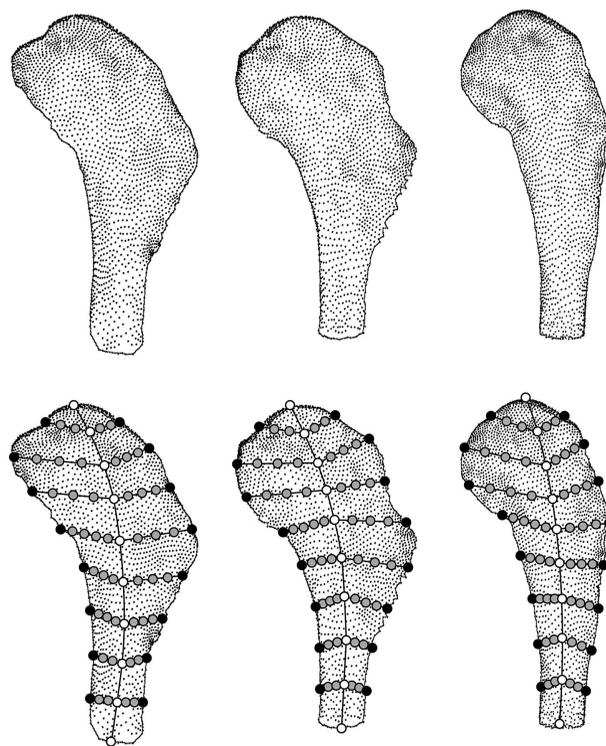


Figure 2.5. Point models of the proximal humeri of diurnal raptors, derived from laser scans (top row), showing the placement of semi-landmarks for eigensurface analysis (bottom row). (From Sievwright and MacLeod 2012.) The species are (left to right) *Falco sparverius*, *Accipiter cooperii*, and *Gyps africanus*. (Copyright: Trustees of the Natural History Museum.)

imaging techniques provide a means to visualize and precisely measure variation in internal traits, including soft and skeletal anatomy, and therefore begin to reveal their relationship to ecological influences at the intraspecific level.

PHYSIOLOGY AND FUNCTIONAL ANATOMY

One of the most spectacular uses of CT imaging of avian anatomy published to date is the study by Düring et al. (2013) on the detailed morphology of the syrinx in the Zebra Finch (*Taeniopygia guttata*). This paper exemplifies how the combination of CT and MRI scans coupled with traditional histology and dissection can reveal anatomical structures and stimulate novel hypotheses about the biomechanical processes that underlie complex behaviors as never before. Another eye-catching example of internal anatomy laid bare is O'Connor and Claessens' (2005) latex cast of the extensive pulmonary air sac system in a typical anatid. The impetus for that study was to extrapolate from avian anatomy to interpret the physiology of dinosaurs, but the image never fails to surprise and impress any ornithologist who has not seen it before.

X-ray videography, in some cases with landmarks in the form of small metal beads surgically implanted, can reveal the internal anatomy in motion in the living bird. For example, Dawson et al. (2011) used this technique to observe the motion of the quadrate during feeding in Mallards (*Anas platyrhynchos*), a motion that had been much written about but not previously studied *in vivo*. Although the action takes place in living birds, such studies rely on specimens to design the research and to interpret the results in an anatomical context. Another biomechanical technique, finite element analysis, is borrowed from engineering and, simply put, models the form under study as a mesh of tetrahedral elements and then uses the model to calculate stresses and strains under different conditions, for instance, to query the biomechanics of pecking in an ostrich skull (Rayfield 2011, Cuff et al. 2015). An intriguing aspect of finite element analysis is its ability to assess the mechanics of alternate morphologies, for instance, by removing a bony process from the skull and asking how the change would affect function (Bright 2014). This method has not been widely applied in ornithology but could prove quite useful in the future.

The aforementioned papers intensively studied one or a few specimens, usually representing a single species, in order to understand avian functional anatomy writ large. Good comparative studies of morphological diversity in the sensory system and organs of birds are also emerging, and these comparative studies tend to make much more extensive use of museum specimens. As examples, Corfield et al. (2015) studied diversity in olfactory bulb size in birds in relationship to potential ecological and behavioral correlates, and Smith and Clarke (2012) studied the association between brain endocast morphology and locomotory mode in Charadriiformes.

EVOLUTION AND DEVELOPMENT

Evolutionary developmental biology is an expanding research area thanks to a suite of improved technologies for studying the genes and mechanisms that influence an animal's development. Laboratory manipulation of model organisms like the chick and the duck continues to be very useful for understanding the embryology of birds (Ealba et al. 2015), but, more recently, a broader, comparative approach is taking hold. The research by Abzhanov and collaborators revealing the developmental mechanisms that determine beak shape in Darwin's Finches (Geospizinae) is a prime example (Abzhanov et al. 2004, 2006; Forster et al. 2008). By examining ontogenetic development in a group like Darwin's Finches, whose ecology has already been intensively studied, Abzhanov et al. were able to integrate knowledge from the two major disciplines of evolutionary developmental biology and ecology, a research interface sometimes termed "eco-evo-devo."

A good review of the modern comparative approach in the field of animal evo-devo is provided by Mallarino and Abzhanov (2012). What is notable for us is that the comparative approach relies strongly on museum specimens. It relies both on establishing the phylogenetic relationships among taxa and on a fine-scale analysis of the morphological differences between them to lay the groundwork for laboratory experiments.

CONCLUSIONS AND FUTURE DIRECTIONS

The instruments, techniques, and computational methods for studying internal vertebrate anatomy are improving in exciting ways. Yet, the internal

anatomy of the bird has been underrepresented in research on avian systematics, evolution, and ecology. This underrepresentation is made clear by the state of avian anatomical and skeletal collections, which are very incomplete compared with traditional study skin collections. For many avian taxa, collections are not adequate for studying the internal anatomical traits of birds, and this is particularly true for studies at the specific or subspecific level. Now is an excellent time for ornithology, as a field, to make up for lost time and to more frequently incorporate internal anatomical variation in research designs that address patterns in ecology and evolution. Enabling further research of the sort discussed in this chapter will require that we build up our skeletal and anatomical collections of birds to meet this new level of demand (see also Chapter 11, this volume). High-quality anatomical specimens, in particular, are in short supply, in part because many older specimens have already been dissected. Emerging research areas, such as comparative evo-devo and neuroanatomy, will require special collections that barely exist now.

In reviewing recent literature on avian anatomy, some noteworthy themes and trends emerge. First, the broadly comparative anatomical papers that are being published using new techniques are often motivated by an interest in interpreting the fossil record. Second, but related to the first point, comparative neuroanatomy and sensory system anatomy are growing research themes, and when these studies broadly survey museum specimens, they too are often motivated by a desire to interpret fossils. Third, ornithological collections are supporting a shift in the field of evolutionary developmental biology toward study of nonmodel organisms, but, to date, only a handful of such studies have been published. We can expect growth in this field and will need to enhance collections to support it. Fourth, functional and biomechanical studies use museum specimens but tend to intensively examine only a few specimens rather than to compare a broad selection. And fifth, some impressive work has been recently published that relates internal morphology to behavior or ecology, but, considering that most practicing ornithologists are primarily interested in behavior and ecology, the amount of work being published in this area is relatively little. Hence, I call specifically upon this large group of ornithologists to think about the untapped potential that lies under the skin of birds.

I would also like to reemphasize that the most common units of comparison in studies of avian anatomy are the species, genus, or family. Ornithology has a deep legacy of research on adaptation and speciation that focuses on variation (often geographic variation) at lower taxonomic levels, such as within species and superspecies or across hybrid zones. I perceive an opportunity to capitalize on this legacy of research by extending it to encompass intraspecific variation in internal anatomical traits, which will lead to studies that better relate anatomy, physiology, and development to the processes of adaptation and speciation. In this sense, we could finally follow the lead of Charles Darwin, who analyzed both internal and external variation in domesticated pigeons, ducks, and chickens to build his case for the evolution of species by natural selection (Darwin 1868).

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