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Guidelines for removal, preservation, and CT imaging of the syrinx, the avian vocal organ

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ABSTRACT—While the state of the art has been described for recording bird songs and calls, there are no described best practices for collecting and preserving the avian vocal organ, the syrinx. In addition to skins and skeletal preparations, field collection of tissues for DNA sequencing has become a common practice. Protocols for such tissue collection often involve accessing internal organs. Here we argue that collecting syringes, which also involves accessing the chest cavity, adds only minimal time to field or skeletal preparation and can be done without damage to the skeleton. Collecting syringes will enable studies of structure–function relationships in the production of calls and song that have not been previously possible. Three-dimensional X-ray computed tomography (CT) imaging techniques allow new insight into these relationships, but material of both male and female birds as well as juveniles is lacking for most avian taxa. Here, we present a guide to the removal and preservation of the avian syrinx and associated vocal anatomy in hopes of increasing their representation in biological collections. In this guide, we suggest that taking in situ measurements is ideal since dissection, fixation, and tissue staining can affect measurements of certain airway features such as tracheal diameter and bronchial length. Our guidelines demonstrate that removing a single wing, shoulder, and half of the pectoral girdle from the dorsal side of the thorax along with disarticulating the sternal and vertebral rib segments on one side of the body allows exposure of the complete airway without damaging the skeleton. We also provide recommendations for fixation, storage, and staining of specimens in preparation for diffusible iodine-based contrast-enhanced computed tomography (diceCT) scans that allow imaging of both soft tissue structures such as vocal folds and muscles as well as the cartilaginous rings that support these structures. *Received 24 January 2020. Accepted 14 December 2020.*

Key words: collections, diceCT, dissection, imaging, morphology, vocal communication.

Instrucciones para la extracción, preservación y generación de imágenes CT de la siringe, el órgano vocal de las aves

RESUMEN (Spanish)—Si bien conocemos los últimos avances para la grabación de llamados y cantos, no hay descripciones de las mejores prácticas para colectar y preservar el órgano vocal de las aves, la siringe. Además de las preparaciones de pieles y esqueletos, las colectas de campo de tejidos para secuenciar su ADN se han convertido en práctica común. Los protocolos para la colecta de dichos tejidos frecuentemente incluyen el acceso a órganos internos. Aquí argumentamos que la colecta de siringes, que conlleva el acceso a la cavidad del pecho, agrega una mínima cantidad de tiempo o preparación esquelética y puede hacerse sin dañar el esqueleto. La colecta de siringes permitirá estudios de las relaciones entre estructura y función en la producción de llamados y cantos que no han sido posibles previamente. Las técnicas de rayos-x tridimensionales de tomografía computarizada (CT) nos permiten una nueva visión de estas relaciones, si bien carecemos de material anatómico de aves machos y hembras de la mayoría de los taxa de aves. Aquí presentamos una guía para la extracción y preservación de la siringe de las aves y la anatomía vocal asociada, con la expectativa de que esta información aumentará su representación en colecciones biológicas. En esta guía, sugerimos que la toma de mediciones in situ es ideal, dado que la disección, fijación y teñido de tejidos puede afectar las mediciones de ciertas características de las vías de flujo aéreo como el diámetro de la tráquea y la longitud bronquial. Nuestras instrucciones demuestran que la remoción de una sola ala, hombro y mitad de la faja pectoral desde el lado dorsal del tórax, junto con la desarticulación de los segmentos de las costillas de esternón y de un lado del cuerpo permiten la exposición de las vías aéreas sin dañar el esqueleto. También proporcionamos recomendaciones para la fijación, almacenamiento y teñido de especímenes en preparaciones para imágenes de tomografía computarizada con base en yodo difuso de alto contraste (diceCT) que permitan ilustrar tejidos suaves como pliegues vocales y músculos, así como los anillos cartilaginosos que dan soporte a estas estructuras.

Palabras clave: colecciones, comunicación vocal, diceCT, disección, imágenes, morfología.

Comparative morphological studies of the avian vocal organ, or syrinx, have a long history (e.g., Vicq d'Azyr 1779, Cuvier 1802). The syrinx has been considered a useful system in avian taxonomy since the 19th century (e.g., Nitzsch 1829, Müller 1847, 1878; Wunderlich 1886, Häcker 1900, Setterwall 1901, Köditz 1925, Ames 1971, King 1989, Prum 1992, Griffiths 1994). More recent comparative studies (e.g., Ames 1971, Warner 1972, King 1989) led to syrinx collections being housed at institutions including the Museum für Naturkunde Berlin, the Yale Peabody Museum of Natural History, and the Field Museum of Natural History. Those collections contain a range of taxa, but they often only sample one sex and employ different protocols for syrinx removal and preservation. Methods for extracting syringes (also

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referred to as "syrinxes") have varied markedly as evidenced by the specimens in those and other major collections (e.g., University of Kansas, University of Michigan, and University of California at Berkeley). Published protocols for removing and preserving syringes appear to be designed for songbirds and other taxa with syringes located close to the tracheobronchial juncture. However, some species have syringes located deep in the bronchi or elaborate tracheal morphologies that have been proposed to be key to shaping sound after it is produced by the vocal organ (Müller 1847, Rüppell 1933, Suthers and Hector 1985, Garcia et al. 2017). The recommended procedures also usually destroy parts of the skeleton (Ames 1971, Cannell 1988).

Ames (1971) proposed a general set of methods for syrinx dissection and preparation. In his protocol, the syrinx is removed together with the bronchi, lower trachea, and a portion of the esophagus through small openings cut through the pectoral muscles, sternum (in smaller birds), and interclavicular air sac. The sternotrachealis muscle (*Musculus sternotrachealis;* henceforth *M. sternotrachealis*) is cut "midway between origin and insertion" (Ames 1971: p. 13). The bronchi are removed via a "careful transverse cut across [their] posterior ends" (Ames 1971: p. 13). Excised specimens are then placed in a solution of 65% EtOH and 10% glycerin for long-term storage. Cannell (1988) elaborated on Ames's (1971) methods. He also calls for removing the syrinx, bronchi, lower trachea ("far enough above the syrinx to include all 'syringeal' features"), and associated esophagus as a single unit (Cannell 1988: p. 290). The thoracic cavity is accessed by first cutting the furcula and cranial edge of the sternum "just to the right of the midline" and continuing the incision through the sternum and breast muscles (Cannell 1988: p. 290). The *Mm. sternotracheales* are cut "near to the thoracic attachment," resulting in more saved muscle than in Ames (1971; Cannell 1988: p. 290). The major arteries are then cut to allow visual access to the syrinx (Cannell 1988). The bronchi are severed "where [they] enter the lung"—Cannell (1988: p. 290) notes that "short bronchi may be the most common deficiency of syrinx specimens." The specimen is then fixed in "10% formalin for several days" and transferred to 70–75% EtOH for long-term storage (Cannell 1988: p. 290).

A review of collections made subsequent to Ames (1971) and Cannell (1988) showed that their protocols have not been broadly adopted. In practice, preparation techniques showed more variation than that captured in these works. But much about their methods is suboptimal even if followed. Most importantly, both authors suggest severing the trachea below the larynx, and neither recommends taking in situ morphological measurements. These lead to loss of data crucial to the study of vocal functional anatomy. Upper vocal tract resonance may play a key role in avian vocal sound production, and soft tissue dimensions are known to change following excision and preservation in fluid (Greenewalt 1968, Perlman et al. 1984, Nowicki 1987, Podos 2001, Riede et al. 2006, Vickerton et al. 2013, Buytaert et al. 2014). Additionally, the syrinx can have vibratory elements in the trachea, bronchi, or the juncture between them (Ames 1971, Warner 1972, King 1989). Tracheal morphology shows similar variety, as evidenced by the convolutions and local enlargements in the windpipes of some anatids, cracids, cranes, swans, manucodes, and other taxa (Latham and Romsey 1798, Yarrell 1827, Heinroth and Koenig 1911, Johnsgard 1961, Fitch 1999). Finally, removing tissues affects their dimensions. The difference between in situ and excised airway diameter measurements is typically small, but bronchial length measurements can decrease by more than 50% following excision. This is because the bronchi are not fully supported by cartilaginous rings. It is always important to minimize compression or stretching of anatomical material during measurement.

We first present a protocol beginning with an unskinned fresh specimen. We then present an abbreviated protocol for cases where the thoracic cavity has already been accessed in the course of skeletal preparation or tissue collection. We recommend combining these preparations with salvage and conservation of the syrinx. In this time of rapid biodiversity change, it is important to use specimens as efficiently as possible—and collecting multiple types of data from the same specimen increases its long-term scientific value (Hendrick et al. 2020, Lendemer et al. 2020). We end with some recommendations for imaging specimens using diffusible iodine-based contrast-enhanced computed tomography (diceCT), an imaging

Table 1. Specimens used during development of syrinx removal and preparation protocols.

Taxon	Common name	Specimen #
Megascops asio	Eastern Screech-Owl	TMM M-16979
Pelecanus occidentalis	Brown Pelican	TMM M-12390
Zenaida asiatica	White-winged Dove	TMM M-13796
Pandion haliaetus	Osprey	TMM M-12387
Nyctanassa violacea	Yellow-crowned Night-Heron	TMM M-16712
Caracara cheriway	Crested Caracara	TMM M-12252

approach increasingly used in studies of soft-tissue anatomy and biomechanics (Gignac et al. 2016).

Methods

The removal and preservation protocols outlined here were assessed for a range of non-passerine specimens of different body sizes and phylogenetic affinities (Table 1). The same methods can be used whether a specimen is fresh or has been frozen and thawed, but the means of treatment and preservation should be recorded as they can affect imaging and measurements. If the specimen will be prepared as a skeleton or used for tissue extraction, we suggest removing the syrinx. In this case, we recommend taking syringeal measurements both in situ and after extraction. In situ measurements are more functionally relevant and will be more comparable to measurements taken from CT scans of full-body staining preparations. However, in general, comparing sets of in situ and excised measurements will introduce error, and in or ex situ measurements may be the only set available for other parts of a clade. With hummingbirds, the body should be fixed and imaged whole due to the location of the syrinx (see below), regardless of whether skin or tissue samples are also taken.

If an entire bird specimen has already been preserved in fluid or fixed whole, dissection of the syrinx will be complicated by rigidity of the tissues. Extraction is not recommended in this case, and digital post-processing of syrinx components from a scanned whole specimen will likely be more time consuming (Li et al. 2015). These specimens can be CT-scanned whole at high resolution using contrast-enhancing agents like iodine (as in diceCT scanning) to study syringeal structure. If the resolution required for scanning the whole specimen is too low to resolve syringeal features, a higher-resolution sub-volume scan containing the regions of interest should be conducted. It is important to note that the duration of staining and cost of scanning increases greatly with body size (Li et al. 2015, Gignac et al. 2016). A dove-sized bird could easily take several months to stain, scan, and de-stain. Phase-contrast CT scanning allows imaging of tissues without staining; it is becoming more available, but remains difficult to access for most researchers (Momose et al. 1996).

All specimens used in the development of this protocol came from the collection of the Jackson School Museum of Earth History (JSMEH) at the University of Texas at Austin. Dissections were conducted at the Vertebrate Paleontology Laboratory of the JSMEH and documented using a DSLR camera; only photographs of Crested Caracara (*Caracara cheriway*, TMM M-12252) are included in the figures.

The diceCT protocols reported below are based on Li et al. (2015, 2016) and Gignac et al. (2016). Several modified protocols were also tested (Table 2) that were developed through a review of diceCT literature and consultation with specialists at the High-Resolution X-ray Computed Tomography Facility at The University of Texas at Austin (UTCT) and the Micro-CT Laboratory at the Museum für Naturkunde Berlin (see Acknowledgments). Staining and scanning of excised tissues were conducted at UTCT.

Results

Fresh specimens

For fresh specimens, the following procedure can be used. **(1)** Skin the bird. Use a scalpel or scissors to cut through the interclavicular membrane and associated connective tissue, opening a window into the anterior thoracic cavity (Fig. 1). **(2)** Remove this tissue and expose the paired *Mm.*

Table 2. Specimens and staining treatments tested during development of diceCT protocols.

Specimen #	Species	Common name	Protocol
TMM M-16695	*Bubo virginianus*	Great Horned Owl	0.5% I_2 (s) in 100% ethanol for 1 week and 1% PTA in 100% ethanol for 4 more days.
TMM M-17012	*Buteo jamaicensis*	Red-tailed Hawk	1% I_2 (s) in 100% ethanol for 1 week and 2% PTA in 100% ethanol for 4 more days.
TMM M-16916	*Fulica americana*	American Coot	0.5% I_2 (s) in 100% ethanol for 1 week and 1% PTA in 100% ethanol for 4 more days.
TMM M-16933	*Leucophaeus atricilla*	Laughing Gull	1% I_2 (s) in 100% ethanol for 1 week and 2% PTA in 100% ethanol for 4 more days.
TMM M-17118	*Ixobrychus exilis*	Least Bittern	0.5% I_2 (s) in 100% ethanol for 1 week and 1% PTA in 100% ethanol for 4 more days.
TMM M-17605	*Bartramia longicauda*	Upland Sandpiper	1% I_2 (s) in 100% ethanol for 1 week and 2% PTA in 100% ethanol for 4 more days.
TMM M-17067	*Meleagris gallopavo*	Wild Turkey	Rinsed in DI water for 20 min. Stained in 1% Lugol's solution for 6 d. Solution refreshed after 2 d.
TMM M-16875	*Mycteria americana*	Wood Stork	Rinsed in DI water for 20 min. Stained in 1% Lugol's solution for 6 d. Solution refreshed after 2 d.
TMM M-16975	*Podilymbus podiceps*	Pied-billed Grebe	Rinsed in DI water for 20 min. Stained in 1% Lugol's solution for 6 d. Solution refreshed after 2 d.
TMM M-16860	*Corvus brachyrhynchos*	American Crow	Rinsed in DI water for 20 min. Stained in 0.5% Lugol's solution for 2 d.
TMM M-17913	*Geococcyx californianus*	Greater Roadrunner	Rinsed in DI water for 20 min. Stained in 0.5% Lugol's solution for 2 d.

sternotracheales on either side of the trachea. They may attach on the sternum, bronchi, or lungs; the attachment site should be noted. Then cut the muscles close to their points of origin, leaving a significant length attached to the trachea (Fig. 1). Note: some taxa (e.g., hummingbirds, *Nothura darwinii*) do not possess *M. sternotrachealis*. In these cases, Step 2 can be skipped. **(3)** Open the abdominal cavity caudal to the ribcage. Remove viscera through this opening. **(4)** Separate the sternal and vertebral rib segments on one side of the specimen (Fig. 2). The joint between the segments can be felt as a small cleft between the 2 rib segments. In many small specimens, the segments can be separated by flexing the joint between the 2 segments and pulling them apart. A small pair of scissors can be used to sever the remaining connective tissue. With larger specimens, it may be necessary to use a scalpel, cutting pliers, or scissors to help separate the rib segments. **(5)** Using a sharp scalpel (and scissors if necessary), cut muscles dorsal to and surrounding the scapula on the same side as the rib disarticulation (Fig. 3). After the cut, half of the pectoral girdle and one of the wings should remain connected to the sternum but be fully disconnected from the dorsal side of the thorax. **(6)** Cut the remaining connections between the heart and the ventral side of the body. Reflect the detached wing, pectoral girdle, sternal ribs, and keel away from the thorax as a single unit. Do not damage the pectoral girdle on the opposite side of the body. **(7)** Excise the heart from the dorsal half of the specimen (Fig. 4). This will expose the entire syrinx and trachea. **(8)** If there is time, we suggest taking the following basic in situ measurements (Fig. 4): tracheal length from the midpoint of the glottal opening to the bifurcation point of the airway; lateral and dorsoventral diameters of the trachea (a) immediately posterior to the glottis; (b) midway between the glottis and the point immediately anterior to the tracheobronchial junction (TBJ); (c) immediately anterior to the TBJ; lengths of left and right bronchi from the TBJ to their entrances to the lung; lateral and dorsoventral diameters of left and right bronchi immediately posterior to the TBJ and anterior to their entrances to the lung. Minimal pressure should be applied during measurement to prevent distortion and damage. If there is extensive tracheal coiling (e.g., Gruidae, Anseranatidae, Paradisaeidae; Heinroth and Koenig 1911, Johnsgard 1961, Fitch 1999) we suggest the trachea not be fully extended for measurement but left in its original position on

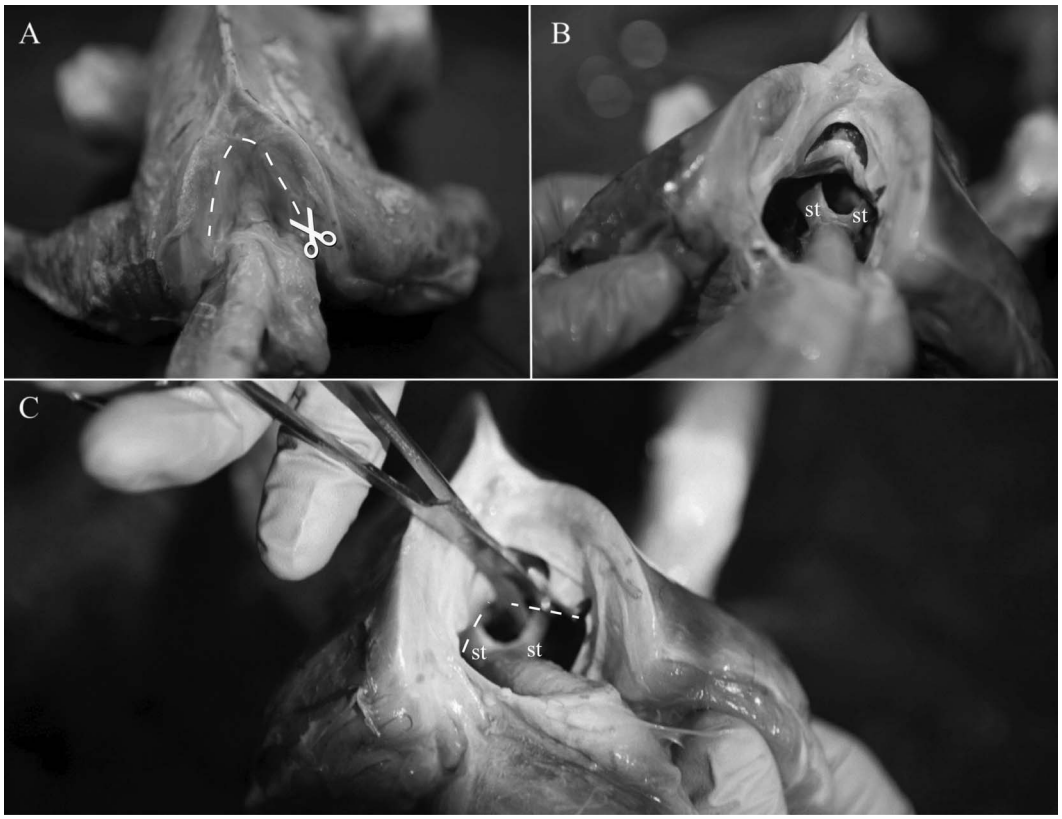

Figure 1. Recommended preparation of the *M. sternotrachealis*, paired muscles that usually connect the airway to the sternum, prior to opening the thoracic cavity. (A) Clear air sac and fascia from the cranial edges of the furcula. (B) Cranial view of furcular opening after removal of the tissue between the rami of the furcula; both *M. sternotrachealis* muscles are clearly visible. (C) Cut the *M. sternotrachealis* as close to the sternum as possible, detaching the *M. sternotrachealis* muscles from the thoracic wall. A significant portion of each muscle should be excised along with the trachea. Abbreviations: st = *M. sternotrachealis*.

or in the sternum and lifted to the side while the syrinx and hyoid are detached. In these taxa, the full vocal tract should then be fixed with the pectoral girdle and thoracic region. If the trachea is too curved or contorted to measure directly with a ruler, a piece of string can be used. **(9)** Use a scalpel to cut the bronchi where they join the lungs (cut marks in Fig. 4). Be sure to excise the entire bronchus, even if it requires taking a small amount of attached lung tissue. If the specimen has an interbronchial ligament, which connects the medial margins of the bronchi caudal to the TBJ (see Fig. 4), take care that it does not become damaged. **(10)** Use a scalpel and scissors to expose the hyoid cornua. Detach the hyoid apparatus with the attached larynx as a unit with the trachea. If you do not wish to remove the hyoid and larynx, cut through the trachea close to the midpoint as indicated in Fig. 4. However, this preparation is not advised as significant information on the vocal tract is lost. **(11)** Use a scalpel to cut the trachea and bronchi from the surrounding tissue. The trachea and esophagus will be firmly attached to one another and attempts to separate them could damage the syrinx in smaller taxa. We recommend excising and preserving them together in such cases. **(12)** Transfer the specimen to 10% Neutral Buffered Formalin (NBF) as quickly as possible. Be sure that the storage jar requires the syrinx to bend as little as possible. After the specimen is fixed, progressively transfer it (3 days in deionized water, 1 day in 25% EtOH, 1 day in 50% EtOH) to 70% EtOH for long-term storage. If 10% NBF is unavailable at the time of the dissection, the

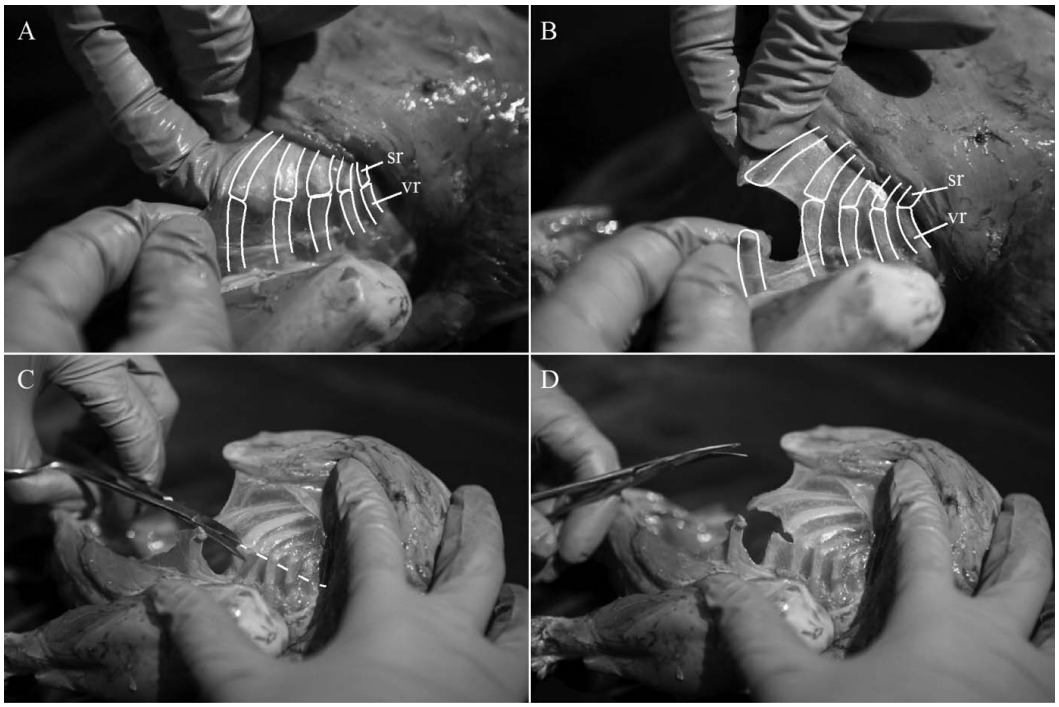

Figure 2. The first step in accessing the thoracic cavity without damaging the skeleton. Two methods of rib segment disarticulation. Upper images (A, B) show a manual approach in small/medium size birds and lower images (C, D) show a tool-assisted approach for larger taxa. (A) Locating the attachment point of the sternal and vertebral rib segments during manual disarticulation. (B) Manually disarticulating the sternal and vertebral rib segments. (C) Locating the attachment point of the sternal and vertebral rib segments during tool-assisted disarticulation. (D) Using scissors to disarticulate the sternal and vertebral rib segments. Abbreviations: sr = sternal ribs; vr = vertebral ribs.

specimen may also be placed directly into 70% EtOH for long term storage, but note that fixing tissues in 70% EtOH generally results in greater shrinkage than fixing in NBF (Li et al. 2016, Perry et al. 2016).

Abbreviated protocol for specimens with an open thoracic cavity

In cases in which an avian specimen has been skinned and the thoracic cavity has already been accessed to retrieve other tissues for genetic data or skeletal preparation, the following abbreviated protocol can be followed. Locate the *Mm. sternotracheales* on either side of the trachea and cut each muscle close to its point of origin, leaving a significant length (at least 3–5 cm in larger taxa) attached to the trachea. If the trachea and bronchi are still attached to the specimen and close to their in situ conformation, take measurements described in Step 8 above. Cut just below each bronchus. Be sure to excise the entire structure, even if you must take a bit of attached lung tissue. Detach the trachea from associated structures, from the bronchi up to the hyoid. Use a scalpel to excise the entire hyoid apparatus, larynx, and tongue. To excise the hyoid, use a scalpel and scissors to expose the hyoid cornua. Then remove surrounding tissue from around the mandible so that the hyoid, larynx, and tongue detach as a unit with the trachea. If omitting the hyoid and larynx, cut through the trachea close to the midpoint as indicated in Fig. 4. Place into 10% NBF for several days to fix. Pick a jar that requires the specimen to bend as little as possible. Progressively step the specimen from DI water to 70% EtOH for long term storage (see Step 12 above). If 10% NBF is unavailable at the time of dissection, the specimen can also be placed directly into 70% EtOH for long term storage (but see note on tissue shrinkage in primary protocol).

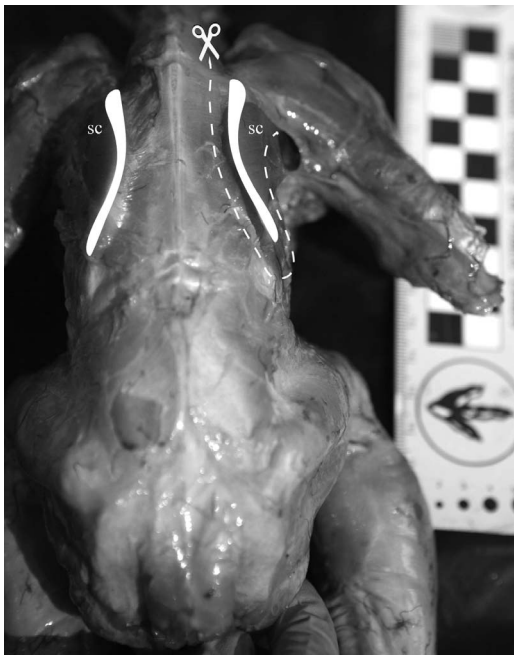

Figure 3. The second step in accessing the thoracic cavity without damaging the skeleton. Disarticulating one of the scapulae by cutting through muscles between the scapulae and the vertebral column. The specimen is shown in dorsal view. Either scapula can be cut along the indicated lines, but only one side should be disarticulated. Abbreviations: sc = scapulae.

Preparing specimens for diceCT imaging

Diffusible iodine-based contrast-enhanced computed tomography (diceCT) allows 3-dimensional imaging of the internal and external morphology of the syrinx including the vocal folds. This approach has been key to forwarding our understanding of the avian vocal organ (Düring et al. 2013, Clarke et al. 2016, Kingsley et al. 2018). The protocols below are for specimens preserved in 70% EtOH. If specimens are preserved in a different solution, begin by rinsing in deionized water and progressively transfer to 70% EtOH. See Gignac et al. (2016) and Li et al. (2015, 2016) for further guidelines. We suggest starting by carrying out a high-resolution μCT scan on the unstained specimen. Contrast between skeletal material and soft tissue can decrease after staining. High-quality unstained scans allow bones and mineralized cartilages to be digitally segmented more easily. Next, prepare the staining solution.

For excised syringes, we recommend using 1% I_2E (w/v) in 100% EtOH (i.e., for every 100 mL of staining solution, dissolve 1 g solid I_2 in 100 mL of pure ethanol). Use of lower concentrations of iodine minimizes shrinkage of soft tissues (Li et al. 2015, 2016; Gignac et al. 2016). Aqueous I_2KI (Lugol's solution) and phosphotungstic acid (PTA)-based solutions are also commonly used for staining soft tissues. Some authors (e.g., Swart et al. 2016, K. Mahlow, pers. comm.) have found that PTA provides better differentiation between tissue types (particularly cartilage), while strong iodine solutions provide the highest overall contrast levels and muscle fiber resolution. A visual comparison of a specimen stained first with I_2E and then with PTA is shown in Fig. 5. It is often necessary to carry out test runs with various staining agents/concentrations/durations to determine which best suits the study requirements. *Note:* Iodine crystals often require several minutes of slow stirring to dissolve. If preparing a large volume of stain, it may be useful to place the solution on an orbital shaker at low speed to assist with crystal dissolution.

One must then estimate staining duration. Gignac et al. (2016: table 1) provides one resource for such estimates based on specimen size and phylogenetic position. For most excised syringes of smaller birds (e.g., sparrow to Rock Pigeon [*Columba livia*] size), 2–3 d is sufficient. Excised material of large species, such as Emu (*Dromaius novaehollandiae*) and Ostrich (*Struthio camelus*), will likely need at least 1–2 weeks and will require repeated refreshment of the staining solution (Li et al. 2016).

When placing the specimen in the staining solution, make sure the specimen is entirely covered and has enough room inside the vessel to avoid folding or other deformation. The specimen should be positioned as close as possible to its in situ conformation. Placing the staining vessel on an orbital shaker at very low speed can enhance the diffusion rate and evenness of staining (K. Mahlow, pers. comm.). To monitor staining, carry out periodic low-resolution (10–20 min in duration) test scans to assess stain progress. Complete the first scan after 2–3 d in stain and then every ~2–3 d thereafter depending on stain progress. Scanner settings for the fully stained specimen will depend on the specimen size, extent of staining, and the type of visualization desired. General guidelines and recommended reporting

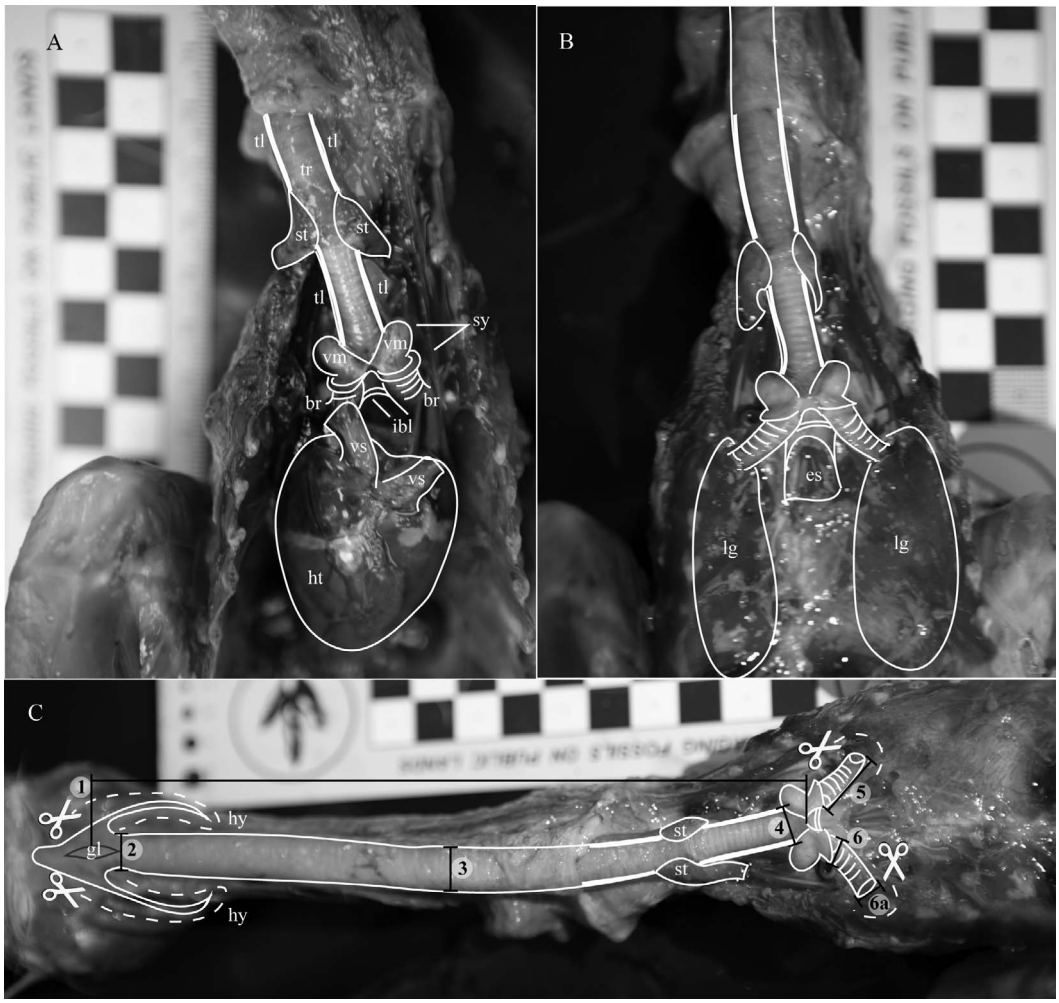

Figure 4. Ventral view of specimen following removal of the pectoral girdle and associated structures. For visual clarity, the whole pectoral girdle is removed here instead of being reflected across the body as indicated in the protocol. (A) includes the heart; (B) is after heart removal. Abbreviations: br = bronchus; es = esophagus; ht = heart; ibl = interbronchial ligament; lg = lung; st = *M. sternotrachealis;* sy = syrinx; tl = *M. tracheolateralis*; tr = trachea; vm = vibratory mass; vs = heart valves. (C) In situ measurements of the trachea, bronchi, and syrinx: (1) tracheal length from just below glottis to the external bifurcation point of the airway; (2) tracheal diameter at glottis; (3) tracheal diameter at ½ of entire length; (4) tracheal diameter just above TBJ; (5) L/R bronchus length; (6) L/R bronchus diameter, just caudal to TBJ; (6a) L/R bronchus diameter, just cranial to lung entrance. White dotted lines indicate suggested cutting paths for bronchi and hyoid removal. Abbreviations: gl = glottis; hy = hyoid cornua; st = *M. sternotrachealis*.

standards can be found in Gignac et al. (2016). To remove the iodine from the stained specimen, transfer specimen to 70% EtOH. Refresh solution every ~2 d until the amount of stain leaching from the specimen appears stable and minimal. Then refresh approximately every week until the solution is clear of color. A low speed orbital shaker can also be used to accelerate the de-staining process. The entire process of staining, scanning, and de-staining a formalin-fixed and ethanol-preserved excised syrinx from a songbird-sized specimen typically takes 2–3 weeks.

Discussion

The evolution of the avian syrinx is an important yet understudied question. New mor-

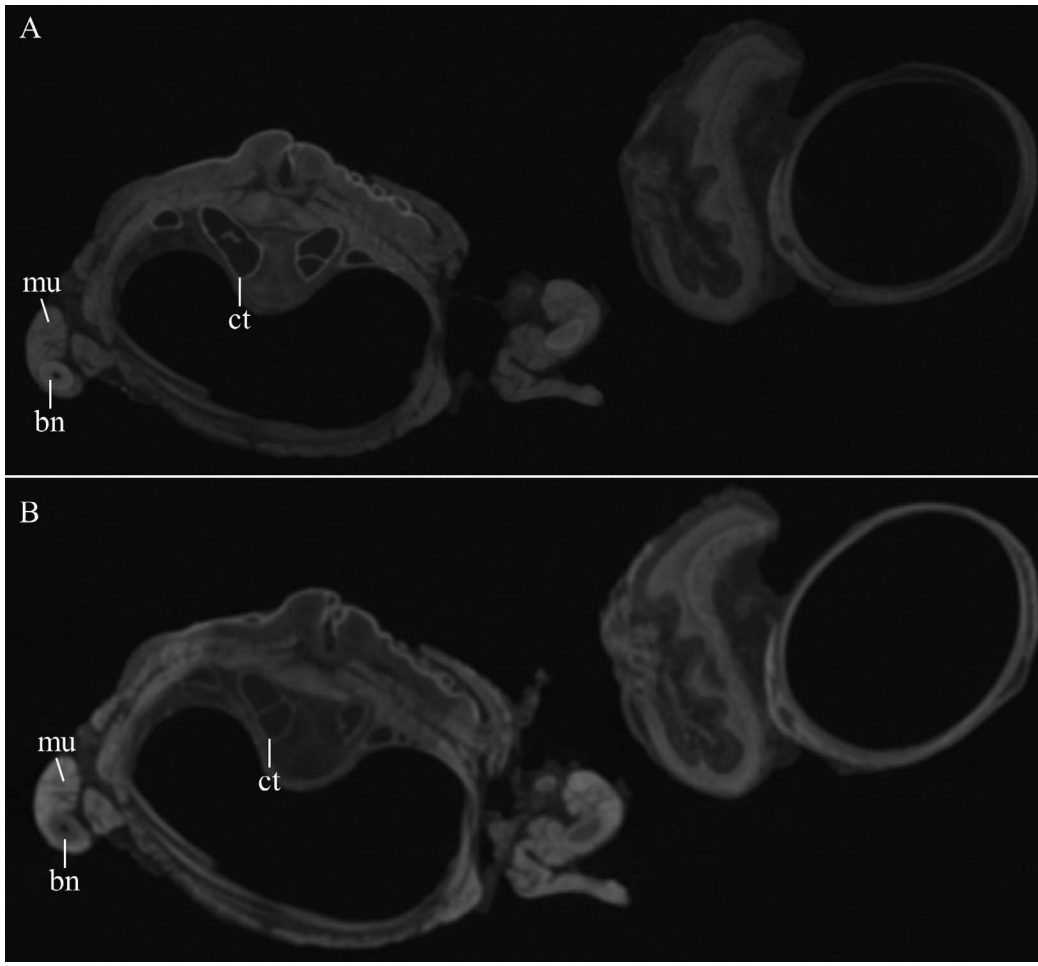

Figure 5. X-ray computed tomography images of an Upland Sandpiper (*Bartramia longicauda*) trachea and esophagus after 2 different staining treatments. First (A), the specimen was stained for 7 d in 1% I_2E solution (1g I_2 / 100 mL 100% EtOH). Then (B), the same specimen was placed in 2% PTA solution (2g PTA (s) / 100 mL 75% EtOH) for 4 additional days. Identical scanner settings were used to create each image. Iodine stains have been suggested to provide optimal overall contrast, while PTA has been found to provide superior differentiation between soft tissue types (Swart 2016); staining agents and protocols must be decided based on experimental aims. Abbreviations: mu = muscle; bn = bone; ct = cartilage.

phological and biomechanical investigations of the organ are held back by a lack of well-preserved specimens in biological collections. We have provided a set of protocols for the removal, preservation, measurement, and diceCT-imaging of avian syringes. The methods described cause minimal damage to skeletal elements and can be conducted relatively quickly in the field or laboratory.

The diceCT imaging guidelines we have provided allow for the generation and broad distribution of high-resolution 3-D morphological data. Staining agents and protocols are being rapidly refined, so it will be essential to keep abreast of new techniques and guidelines as they develop. It is also important to highlight that staining durations can be highly variable based on specimen size, preparation, and composition. Tissues of interest should be determined ahead of time and their staining progresses should be regularly assessed via short test scans.

We hope that this guide will help drive an expansion of syrinx holdings in academic institutions. Comparative studies on syrinx morphology

and function will shed much needed light on the evolution of the avian voice.

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