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A Method for Removing Eggs or Embryos from Preserved, Gravid Snakes that Minimizes Damage to Museum Specimens

Christopher Sheil

Luke J. Welton

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An impressive number of studies utilize the eggs and embryos of model organisms (e.g., *Danio rerio, Gallus domesticus, Mus musculus, Xenopus laevis*, and *X. tropicalis*) to evaluate developmental genetics and morphology. Because these species are common, amenable to life and reproduction in laboratory conditions, and often reproduce rapidly and yield large numbers of offspring in a single event, methods of acquiring eggs and embryos from these species are relatively well established and routine. However, beyond these model organisms, far fewer studies utilize eggs/embryos from species that are rare, elusive, or not amenable to life in a laboratory. This is particularly true for reptiles, which often produce far fewer eggs per cycle of reproduction than do most amphibians and fishes, and for which it is often difficult to acquire gravid females if a species is uncommon, narrowly distributed, or elusive (Clark 1937). Studying development in species such as these requires access to embryos at various points in development to capture snapshots in time, and typically requires access to sufficient numbers of specimens to document intraspecific variation. If live specimens are not available from which to collect appropriate material, other methods of sampling embryos are required. Previously-collected, formalin-fixed and alcohol-preserved museum specimens of ovoviviparous snakes, collected from throughout the range of a species and on various dates, represent a source of embryos that can be used for developmental morphology and studies of anatomy (in oviparous species, most of the embryonic development occurs after eggs have been lain). However, use of specimens in museum collections requires consideration of competing perspectives that must be weighed in the context of the value and merits of the research benefits to be gained: 1) ensuring long-term persistence of every specimen and preventing unnecessary damage to this material; 2) maintaining linkages between specimens and their associated data; and 3) making specimens available for research that expands our knowledge about a particular taxon, generalized processes of evolution or development, or cross-disciplinary inferences. To balance these perspectives, methods should be used that minimize damage to museum specimens (Simmons, 2014:81, and references therein) when cutting or altering material is required. Additional consideration should be given to paired organs (e.g., oviducts) to ensure that one remains intact for future studies. Herein, minimally destructive methods are presented for removing eggs/embryos from formalin-fixed, alcohol-preserved, gravid ovoviviparous snakes. A recommendation is also made for placement and type of incision to be made in snakes that are to be preserved and deposited as voucher material in a museum collection.

To document the feasibility of using historical museum specimens as a source of snake embryos, a survey of specimens in the herpetological collection at the University of Kansas Biodiversity Institute was conducted, and found that of 2965 specimens of three species of *Nerodia*—*N. erythrogaster* (N = 543), *N. rhombifer* (N = 398), and *N. sipedon* (N = 2024)—at least 63 (= 2.12 %) were gravid and in stages of reproduction in which eggs and embryos could be easily confirmed by manual palpation. The majority of these gravid specimens had pre-existing incisions in the ventral or lateral body wall, likely for reasons that included to: 1) expose viscera to fixatives and/or preservatives; 2) examine gut contents; 3) examine specimens for endoparasites; 4) remove tissue samples for necropsy or biochemical/genetic studies; and/or 5) confirm reproductive condition of an individual at the time of death. Many of these incisions caused moderate damage to scales, epidermis, underlying musculature, and internal organs (e.g., oviduct, intestine, stomach, liver), suggesting haste or a lack of attention to detail when these original incisions were made. Cuts ranged from relatively short (spanning only one or a few ventral scales) to those that spanned nearly the entire length of the animal.

Methods Involved

Sexual dimorphism in tail anatomy is conspicuous in snakes (Powell et al. 2016:figure 169; Fig. 1A and B) and gravid females can be identified easily by palpating the ventral body wall in a zone corresponding to the posterior one-third to half of the body, anterior to the vent (Fig. 1B). Fitch (1987) describes proper methods for palpating live, gravid females; the technique is similar for preserved snakes in that the specimen is grasped around the body in one hand with the belly-side facing up, and the belly is palpated with the thumb of the opposite hand to feel within the oviduct for follicles, eggs, or embryos. With practice and

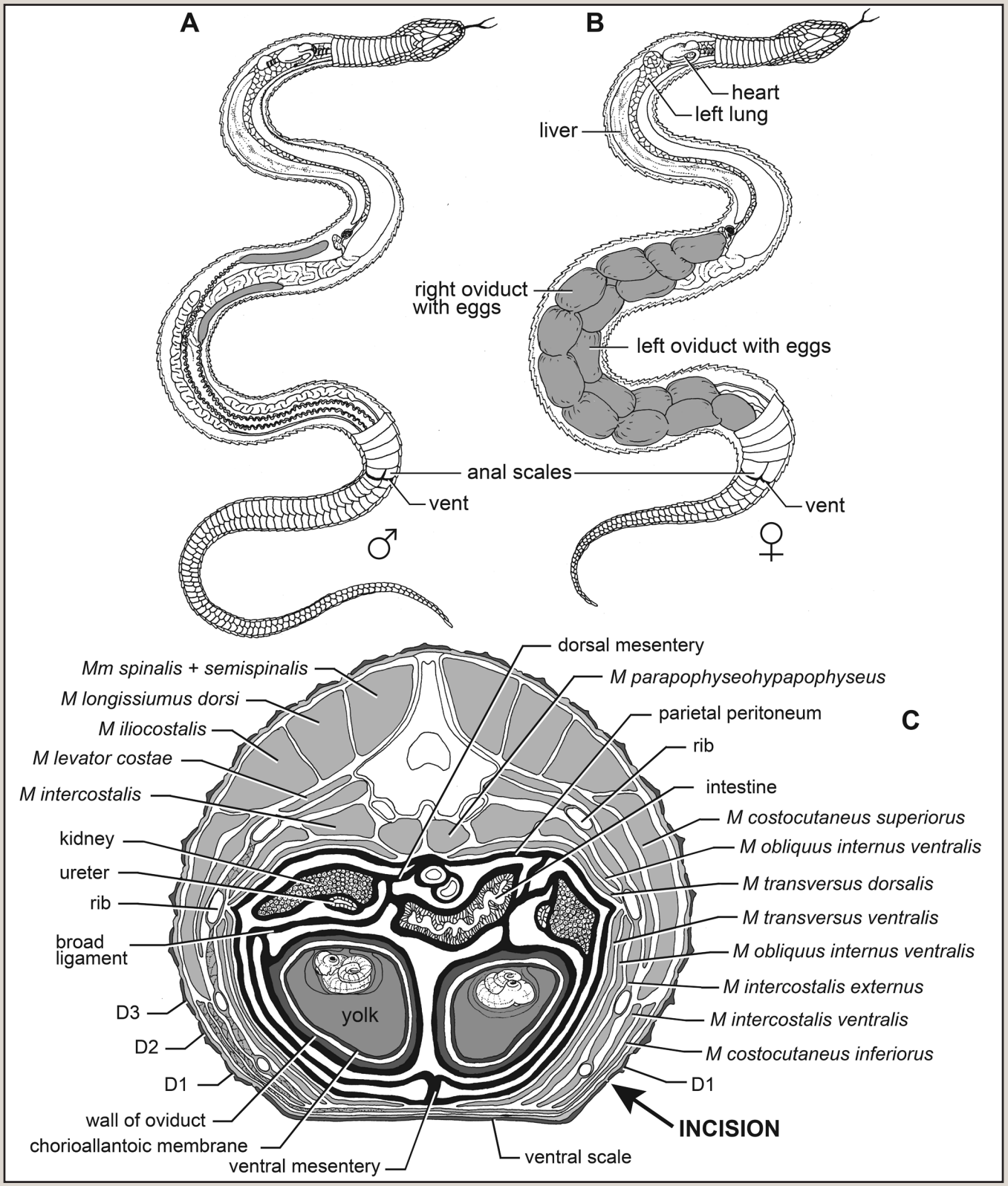

FIG. 1. Sexual dimorphism, general anatomy, and relative position of eggs/embryos in gravid female snakes. A) ventral view of male snake with tail that is relatively longer and tapering more gradually towards the tip (to accommodate inverted hemipenes) than that of a female. B) ventral view of female snake with tail that is relatively shorter and tapering more abruptly towards the tip. In females with eggs/embryos approaching maturity, the oviduct and eggs will occupy the posterior half to one-third of the coelom, anterior of the vent; during later stages of development, this region will exhibit greater girth and the number of eggs can be estimated with reasonable accuracy by palpating. C) generalized cross section of gravid female at mid-body of oviduct with mature eggs/embryos, showing layers of tissue and organs that will be encountered. Arrow indicates preferred position of new incisions, between ventral scales and D1. Thick black lines indicate relative position (but not thickness) of peritoneum and mesenteries, which will be thin and translucent. Terminology for muscles from Gasc (1981). D1, D2, D3 = first three dorsal scale rows.

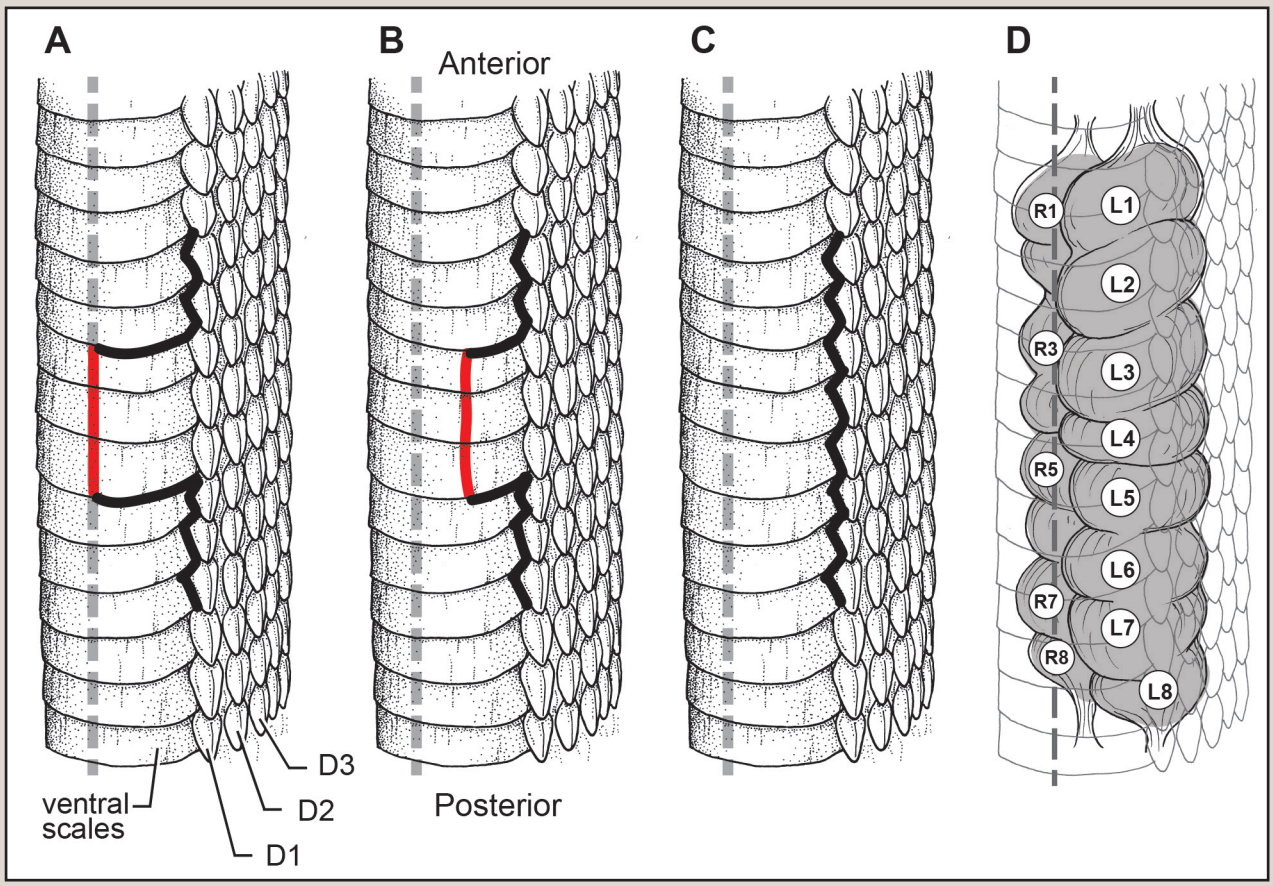

FIG. 2. Left ventrolateral view of body wall of gravid snake showing arrangement of scales and preferred placement of incisions. Pre-existing incisions likely will exist in ventral scales at midline (A) or slightly off the midline (B); in these instances, incisions that require expanding can be made larger by cutting between neighboring ventral scales towards the larger (i.e., outer) curve of the body, and then cutting either anteriorly or posteriorly between D1 and ventral scales in a zig-zag pattern between these scales. C) If a pre-existing incision does not exist near the desired region of entry, place any new incisions between the lateral margin of ventral scales and ventral margin of first row dorsal scales (D1), which will require following a zig-zag pattern between these scales. D) smaller incisions made in a zone corresponding to the middle half of the length of the oviduct (determined by palpating the gravid female) will provide access to eggs/embryos directly below the incision (e.g., L4 and L5), as well as several neighboring eggs (e.g., L3 and L6) by working cranially and caudally within any existing incision; this will afford access to the greatest number of eggs/embryos with the least damage to a cataloged voucher specimen. Thick black line = location of new incision; red line = location of pre-existing incision; dashed gray line = midline of ventral scales; D1, D2, D3 = first three dorsal scale rows; L & R = left and right oviducts, respectively.

experience, it is possible to distinguish items in the oviduct from those in the stomach (which is located more anteriorly) and intestine (which typically present a different firmness than do the eggs or embryos). Even in large, heavy-bodied, muscular snakes (e.g., *Nerodia rhombifer*) that are stiff from formalin-fixation and alcohol-preservation, palpation can be used to estimate the approximate number of eggs/embryos within a gravid female. For example, egg counts taken by palpation of large- and small-bodied preserved specimens of *N. erythrogaster, N. rhombifer,* and *N. sipedon* were not different from counts taken from the same specimens via radiography (2-tailed paired t-test; N = 26; df = 25; P = 0.898). Digital radiographs of these females were taken with an INSPEX 20i Digital X-Ray Imaging System (Kodex, Inc., Nutley, New Jersey) using a TFI Picker "Hotshot" 110 KV portable X-Ray Unit (Diano Corporation, Tucker, Georgia) and a Mars 1417V Innovative Wireless Cassette-size Detector (iRay Technology, Shanghai, China) (3–4 second exposure; 5 mA; 35–48 KVp, depending upon size of specimen) and processed with Merlin Mobile X-ray Imaging V1.10.1. However, practice may be necessary to recognize instances in which late-stage embryos retain relatively large masses of yolk, which will be easy to confuse as separate eggs or embryos, thereby artificially increasing the estimated counts of available specimens.

The anterior- and posterior-most extents of the oviduct (Fig. 1B) can be determined by palpating eggs within a gravid female. After the position and general number of eggs has been identified, the specimen is examined for previously made incisions that can be expanded to provide access to eggs in one of the oviducts. Common museum practice is for dead snakes to be neatly looped or coiled during hardening/formalin-fixation and preservation in alcohol (Simmons 2014, 2015) so that they fit more easily into jars and tanks; therefore, most specimens will expose either the right or left side along the outer (i.e., larger) curve of the body. To increase space within which to work while removing eggs/embryos, and to minimize damage to specimens, it is preferred to work along this exposed, larger curve of the body. Additionally, when possible, it may be preferred to expand upon pre-existing incisions, as this will minimize damage to specimens by utilizing pre-existing access to the coelom. If pre-existing incisions are present in the ventral scales corresponding with region of the oviduct (Figs. 2A

and B), use narrow-tipped scissors to cut the skin transversely (i.e., laterally) between neighboring ventral scales towards Dorsal Scale Row 1 (D1; Figs. 2A and B), at which point incisions will be made anteriorly or posteriorly between the lateral margin of each ventral scale and the ventral margin of each scale in Dorsal Scale Row 1, following a zig-zag pattern. Cutting the skin at this location avoids damage to scales that may be useful in future studies and creates an incision that appears clean and lays easily back in place once the procedure is complete (thereby holding viscera and muscles neatly within the specimen). If pre-existing incisions are not present within the region of the oviduct, a new incision can be made by carefully cutting with narrow-tipped scissors between the lateral margin of the ventral scales and ventral margin of D1 (Fig. 2C), following a zig-zag pattern between these scales. In each scenario, holding the scissors at a relatively shallow angle (< 20° relative to the surface of the skin) guarantees that only the skin is being cut and minimizes unintentional damage to underlying tissues. These methods mirror those of taxidermists, yielding the added benefit of effectively disguising incisions and maintaining the overall integrity of external morphological characters. If necessary to separate skin from underlying tissues, a poke-and-separate technique can be used whereby the closed tips of the scissors are inserted gently between the skin and underlying musculature, opening the scissors to spread the layers apart, and then snipping the freed skin. The sufficient length of the incision is determined by the size of the animal and amount of space required to insert forceps, scissors, and (possibly) fingers to remove eggs/embryos. Once the skin has been cut, a small incision is made through all underlying muscle layers (*Mm costocutaneus inferiorus, intercostalis ventralis, intercostalis externus, obliquus internus ventralis,* and *transversus dorsalis*) and parietal pleura to open the coelom (Fig. 1C). Grasp the trunk musculature at the margin of the cut with forceps, and with intentional, straight cuts expand the incision anteriorly and posteriorly to expose viscera; hold the scissors at a shallow angle to ensure that viscera are not damaged before they are visible. Hemostats clamped to the skin and muscle on one or both sides of the incision are recommended for holding the body cavity open while cuts are made through thicker layers of muscle in larger specimens, and are ideal for holding the incision open while examining the oviduct and eggs/embryos, particularly when it is necessary to insert forceps, scissors, and fingers into a confined anatomical space.

Because of the requisite to maintain as much of the original integrity of museum specimens, efforts should be made to err on the side of making smaller incisions to minimize the damage caused by dissections. To access the greatest number of eggs/embryos in a female while causing the least damage to the specimen, remove samples from the mid-body of the oviduct (determined by palpating the gravid female), where there is access to those directly adjacent to the incision (e.g., L4 and L5; Fig. 2D), as well as several neighboring eggs (e.g., L3 and L6) that are positioned anteriorly and posteriorly within the incision. All eggs/embryos within a gravid female are at approximately the same stage of development (Velhagen 1995). If a large number of eggs/embryos are required, remove samples from several smaller cuts, rather than one larger cut along the body. Unless they are excessively long, cuts along the midline provide relatively less space in which to remove eggs.

Depending upon the reproductive condition of the female and the quantity of yolk within follicles or eggs, the oviduct and eggs will be conspicuous within the coelom. Visceral peritoneum covering the oviduct is extremely thin, and the walls of the oviduct, though fibrous and tough, are translucent and provide easy visualization of the individual eggs (Figs. 1C and 2D); each oviduct is held in place by the broad ligament (Fig. 1C). If this procedure is being used to assess the degree of development of eggs/embryos, note that the embryo will reside on the dorsal pole of the egg (Stewart and Brasch 2003), within a depression in the dorsal surface of the yolk (Fig. 1C). Therefore, in ovoviviparous species it will be necessary to lift and rotate one or more eggs to view the dorsal surface of the yolk; it may be necessary to remove small portions of yolk to inspect the embryos and determine developmental stages (e.g., Zehr 1962).

To retain greatest integrity of vouchered specimens, remove only some eggs/embryos from one oviduct in any female specimen, leaving the other intact for future research. To remove an egg, create a small incision through the visceral peritoneum and oviduct along the exposed ventral surface of the egg, being careful not to slice into the shell (oviparous species) or chorioallantoic membrane and yolk/yolk sac (ovoviviparous species). The egg is removed by spreading the oviduct around the egg with one pair of forceps while grasping the egg gently and pulling upward and out with a second pair. Each egg can be removed through a single short incision in the oviduct, thereby leaving other portions of the oviduct intact and reducing the chance for any remaining eggs to fall out of the oviduct (and body) during curation or future examination of the specimen. Detailed notes should be taken to document the position of each egg and the oviduct from which it was removed (see Fig. 2D); adequate note taking should be used to document which egg/embryo/pup came from which female snake, as well as which oviduct. To offset specimen data that will be lost by cutting and removing embryos, data should be entered into specimen catalogs and databases (Simmons 2014), including notation about the: number of embryos removed from a gravid specimen; oviduct from which the eggs were removed; position of individual eggs within the oviduct; developmental stage of any embryos (e.g., Zehr 1962); and mass or SVL/TL of any embryos.

Considerations for Field Preparation, Fixing, and Preserving Gravid Snakes

If it is necessary to open recently dead snakes to expose viscera for formalin-fixing and preservation, make these incisions between the ventral scales and Dorsal Scale Row 1, within the region of the oviducts along the posterior one-third to half of the body, particularly for gravid snakes. These incisions will allow fixative and preservative fluids to penetrate the viscera (including eggs/embryos) and muscles as easily as midline incisions used in traditional methods for long-term storage, but will also afford greater access to reproductive anatomy and eggs/embryos for future research, and will yield no greater damage to the specimens beyond that which results from typical specimen preparation. It may be ideal to make these cuts in the skin and body wall prior to hardening specimens with formalin, when the specimens are more pliable and when neater cuts could be made with less damage to neighboring tissues. Admittedly, these methods will be easier to apply with larger snake species (e.g., *Boa*, *Crotalus*, and *Nerodia*), than for smaller snake species (e.g., *Storeria*, *Tropidoclonion*, and *Virginia*). Practicing these methods likely will require greater attention to detail and may take more time to implement, but the tradeoffs in time spent preparing specimens will be outweighed by the need for long-term care and management of museum specimens. These methods are not meant to hinder or discourage studies based on museum specimens, but rather are suggested to

provide practices that minimize damage to voucher specimens of rare and common species, and ensure their long-term integrity.

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