Technical note: A technique to mount Sarcophagidae and Calliphoridae (Diptera) larvae for forensic identification using geometric morphometrics

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A B S T R A C T

Geometric morphometric analyses use landmark coordinates from images of specific areas on specimens. Therefore, to minimize differences arising from human preparation, preparation of the specimen is crucial. The application of geometric morphometrics has been used in entomology for identifying adult dipteran species, however, applying this technique to larval specimens requires the production of standard protocols which would allow specimens to be compared equally. This proposed protocol consists of a two-step process:

1. using 15% potassium hydroxide (KOH) to clear the specimen, followed by rinsing the specimen with 80% ethanol;

2. removal and spreading of the mouthhooks on a slide for imaging and analysis.

The goal of this work is to standardize specimen preparation, which would reduce human error and facilitate use of geometric morphometrics to identify dipteran larvae.

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1. Introduction

Current dipteran keys that include forensically important larvae [e.g. − 1–3]: 1) do not provide comprehensive larval species coverage; 2) can be difficult to work through; and 3) offer no statistical support for decisions. These downsides indicate that there is a need for an improved method of larval identification. Recently, Nunez and Liria [4] demonstrated a high degree of statistical support for the species separation of larval Calliphoridae specimens using the cephalopharyngeal skeleton and geometric morphometrics. We support Nunez and Liria's enterprise as an excellent path forward and herein present a protocol that improves on their initiative by addressing a major problem in geometric morphometrics: specimen axial alignment relative to the plane of analysis.

The cephalopharyngeal skeleton is widely used in larval identification given the profound shape differences between species [e.g.— 1, 3, 5, 6]. The cephalopharyngeal skeleton, especially mouthhooks, has a huge advantage over other larval structures in that they offer the most consistently hardened and clear-edged structures in the larval life stages, making these features ideal for preservation and geometric morphometric analysis. Mouthhooks in particular have hard edges, allowing for clear decisions about landmark placement, whereas other portions of the cephalopharyngeal skeleton, such as the pharyngeal sclerite, ventral and dorsal cornu, have feathered edges that only allow for semi-landmarks to be placed. There are differences between landmarks and semi-landmarks, that can have significant impact on geometric morphometric analysis [7].

The hard edges of the cephalopharyngeal skeleton allow for clear delineation of structures to create shapes for geometric analysis, except when the natural bilateral symmetry of the structures occlude the true shape of the structure by creating confusion of where one side begins and the other ends. We diagram this problem in Fig. 1, and explain it in more detail below.

In geometric morphometrics there is a crucial need for undamaged specimens as data is collected from pictures or scans of the specimen [8]. Organisms with bilateral symmetry, when viewed from the sagittal plane, need to be in perfect perpendicular alignment to the viewer (or camera) in order to accurately reflect the true shape of the structure in question—to be presented undamaged in shape. This configuration is presented in Fig. 1-A, and

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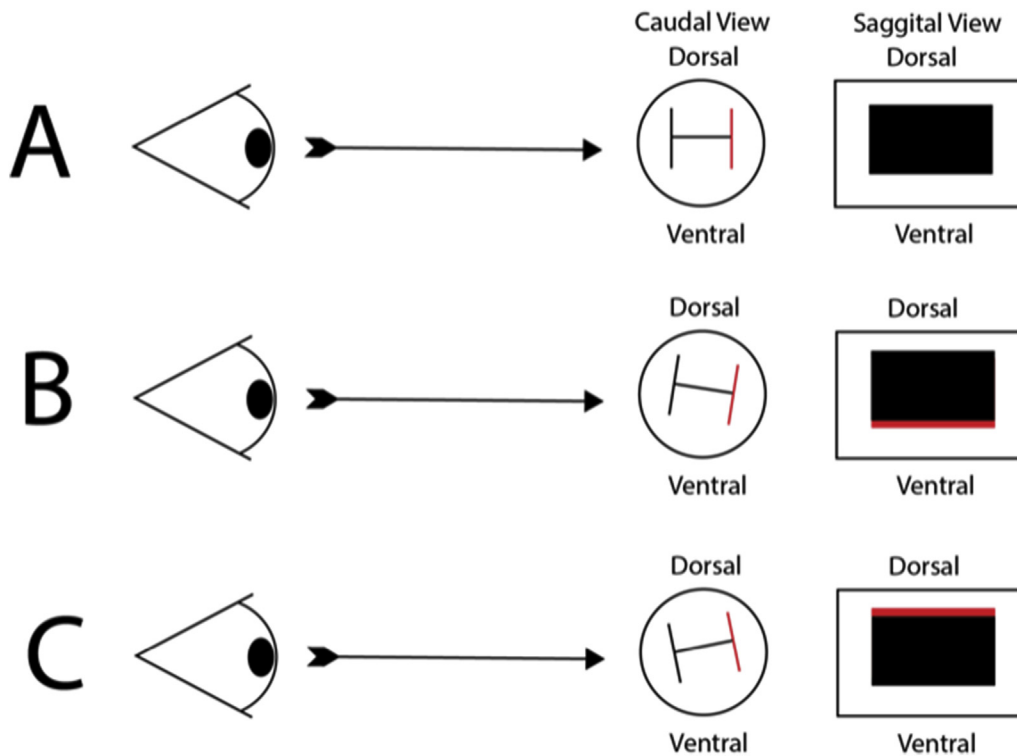
Fig. 1. Schematic demonstrating the problems with axial tilt in bilaterally symmetrical organisms, such as dipteran larva, when visualizing structural outlines from a saggital plane for the purposes of geometric morphometrics. Example A is viewed from a true perpendicular angle, and relates correct structural detail. Example B and C have skewed *bauplans* relative to the viewer, which introduces morphometric error due to either (1) adding the left and right structures together, or (2) confusing the left and right structures when viewed from a perpendicular angle.

demonstrates that the sagittal view in this instance accurately presents the structure to the viewer. In Fig. 1-B and 1-C, the *bauplan* of the organism is skewed slightly *vis-à-vis* the viewer and plane of analysis; in doing so, the sagittal view no longer accurately reflects the true shape of the structure in question. For fly larvae this problem is an artifact of retaining the structure inside the organism while attempting to utilize it for shape analysis, and we can find no practicable way to accommodate this problem while retaining the structures inside the organism. The problem is widespread and is demonstrated in the blue and red arrows of our Fig. 2. This same axial artifact can be seen in Nunez and Liria's Fig. 1—it is likely that the softening of cephalopharyngeal edges in this figure is actually photographic evidence of the paired symmetrical structure being out of focus and creating a halo effect. Further, in Fig. 1-C from Nunez and Liria, you can clearly see the confusion caused by the paired structures in point 2. Is point 2 on the left or right structure, and how does that change the morphometrics of the shape in analysis relative to the other points? This is a form of shape "damage", and our technique clarifies these problems so that un-ambiguous points may be chosen.
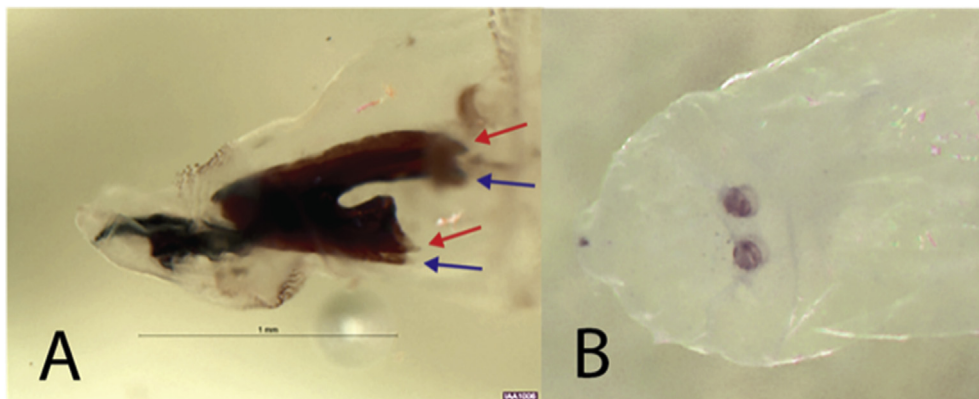

Fig. 2. Third instar *Phormia regina* larva cephalic (A) and caudal (B) ends, cleared using this protocol. Note the blue and red arrows at the cephalic end, which demonstrate the problems with orienting the cephaloskeleton while it remains inside the organism. It is not possible to tell which of the terminal structures are in which plane, of even if the two sets of red or blue arrows each represent the same planes within each colored grouping. In this case, while the overall structure is clear, this specimen could not be utilized for geometric morphometrics due to overlaps between the left and right symmetries. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Undamaged specimens are defined as specimens with no physical alteration or visual obstructions that would make the analysis of shape impossible. Damaged or disfigured specimens alter shape, corrupting the data. Species uniqueness of the data should stem from the underlying shape of the larval mouthhooks—it should not be changed by collection and/or preparation techniques.

Several papers detail procedures for clearing and/or mounting specimens [4,9−14]. Described methods include differences in: 1) temperatures; 2) whether the specimen is whole or partially mounted; 3) type of clearing solutions; and 4) concentrations of the clearing solutions. All of these factors can impact the shape of the cleared specimen. Geometric morphometrics requires specimens to remain effectively still to prevent movement and, therefore, distortion and displacement when placed on a microscope slide, yet the specimen must also be somewhat malleable to prevent breakage and feature loss while preparing the slide. For this reason, whole mount techniques like those proposed by Richet et al. [1] are unsuitable for geometric morphometrics because you cannot clearly see the true shape of the cephalopharyngel skeleton reliably. Rather, specimens should be cleared and the cephalopharyngel skeleton removed for clarity and standardization. Barbosa et al. and Szpila [14,15] describes how to cut and spread muscamorpha larvae for identification, building off the work of Ferrar [5]. Richet [13] mentions dividing the cephalopharyngeal skeleton, but offers no advice on how to flatten the three-dimensional structure of the skeleton as is necessary for geometric morphometrics. Without an expensive technique such as micro CT scanning [16] the three-dimensional structure cannot be properly and reliably oriented *in situ* in a consistent manner for accurate geometric morphometrics analysis.

The use of geometric morphometrics in entomology was reviewed by Tatsuta et al. [17]. Although published research on the shape analysis of wings and genitalia of adult flies are plentiful [e.g. 18−20], geometric morphometrics using the acephalic cephalopharyngeal skeleton of muscomorpha larvae is limited to the work of Nunez and Liria [4], which does not include a standard protocol for ensuring shape conformation, rather, relying on techniques like those presented by Ferrar [5]. Here we establish a protocol for the clearing, flattening, and mounting of sarcophagid and calliphorid larval mouthparts for subsequent use in two-dimensional geometric analysis of muscomorpha larvae. We expand and modify published procedures to include the splitting of the left from right sides of the cephalopharyngeal skeleton with a dorsal cut through the epistomal plate and intermediate sclerite in order to divide and spread the cephalopharyngeal skeleton. This allows for the organization of the mouthhooks and associated intermediate, accessory oral, dental, and pharyngeal sclerites in such a way as to allow for shape analysis. Our process utilizes easily obtainable, cheap materials, and ensures all relevant cephalopharyngeal features are preserved when mounted on a slide.

2. Materials and methods

We developed this method using samples from two different families of forensically important flies: first using 3rd instars of *Sarcophaga bullata* (Sarcophagidae; Parker, 1916) that were obtained from colony, and second using 3rd instars of *Phormia regina* Meigen (Calliphoridae; 1826) and *Lucilia sericata* (Miegen, 1826), reared from wild-caught adults gathered in West Lafayette, Indiana or Davis, California. The *S. bullata* larvae were selected for the first trial as they are much larger in size and easier to handle than *P. regina* or *L. sericata* larvae. All larval morphological nomenclature follows that of Teskey [21], except for the intermediate sclerite, where we follow Ferrar [5].

2.1. Specimen preparation

Adults of all three species were maintained on a diet consisting of sugar (Dominos, Sugar-Pure Cane Granulated) and water, *ad libitum,* in a 30.5cm x 30.5cm x 30.5cm colony box (Bioquip, Collapsible Cages 1450B). Beef liver (Purdue Butcher Block) was used as a medium for oviposition and as a food source for resulting larvae. Third instars with empty crops (prepupal, or wandering stage) were placed in boiling water for 1 min and then preserved in ~80% ethanol until being cleared. Specimens were stored in ~80% ethanol, in case we wanted to use specimens for molecular work as well. We normally cleared specimens the next day, but in our experience, specimens stored up to several months did not pose a problem with this technique. Specimens were prepared for clearing by making a 2−3mm longitudinal incision on the ventral side between abdominal segments III through V (cut 1 from segments aIII-aV; Fig. 3).

2.2. Clearing

Prepared specimens were placed in a 150 ml flask with a 15% potassium hydroxide (KOH) aqueous solution for 24 h at room temperature (~23 °C). To ensure penetration of KOH, the flask was agitated by hand for approximately 30 s, 3−4 times over 8 h. A laboratory shaker would work just fine for this process. After 24 h the entire cephalopharyngeal skeleton was visible from the outside of the specimens through the cuticle (Fig. 4); any remaining opaque gut content or fatty tissue was easily removed during subsequent dissection. The larvae were removed from the KOH solution and rinsed for 1 min in a bath of 100% ethanol, before being placed in~80% ethanol for storage until dissection. Although the dissection can be done without clearing the specimen, it is much more labor intensive and more likely to lead to specimen damage, as the dissector must scrape away the tissues surrounding the mouthhooks. Clearing the specimen with KOH is destructive in nature, and care must be taken to observe the specimens while they are macerating in solution. It is important to halt clearing at a time when the soft tissues are largely removed and clarified, but the structures of the cephalopharyngeal skeleton have no observable damage.

2.3. Dissection

Once cleared, specimens were prepared for slide-mounting, which first requires dissection. To dissect the specimen:

1) Extend the initial incision on the ventral side toward the posterior end through segment aVII and towards the anterior end through thoracic segment II (Cuts #2 & 3 in Fig. 5).
2) Using a sharp scalpel, disconnect the posterior end just past the seventh abdominal segment (Cut #4 in Fig. 5).
3) Similarly, disconnect the anterior end between the first and second thoracic segment (tI&tII), taking care not to slice through the cephalopharyngeal skeleton. (Cut #5 in Fig. 5).
4) Remove the cephalopharyngeal skeleton by pulling it out of the incision made by cut #5 using scalpels and micro forceps (Bioquip, Micro Dissection Kit 4761) to separate the connective tissue. This leaves the remaining portion of segment tI intact.
5) Cut the cephalopharyngeal skeleton through the epistomal plate, dorsal bridge, and intermediate sclerite starting from the dorsal side to allow the sides of the skeleton to be divided and spread on the slide (Fig. 6). The cephalopharyngeal skeleton will be fragile and should be handled gently to avoid damaging the important lateral segments. NOTE: this will damage the
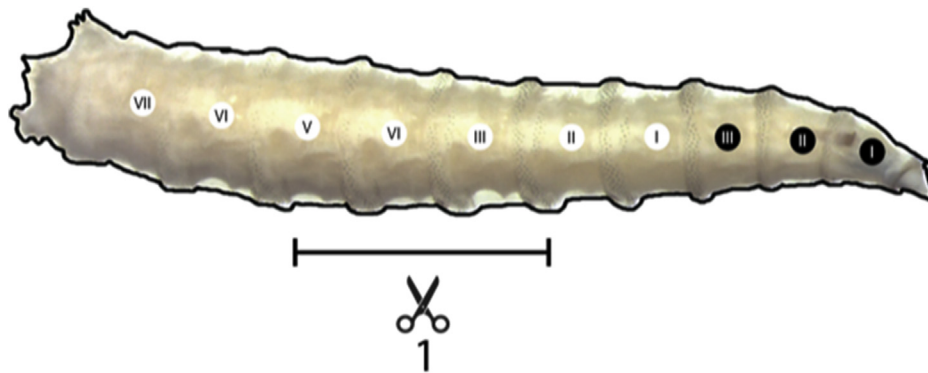
Fig. 3. Dipteran larva illustrating the location for the first cut. Where thoracic segments are denoted by black circles, and abdominal segments are denoted by white circles.
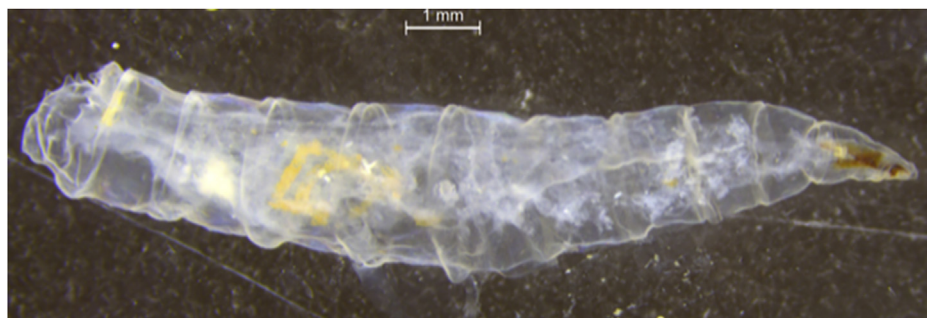

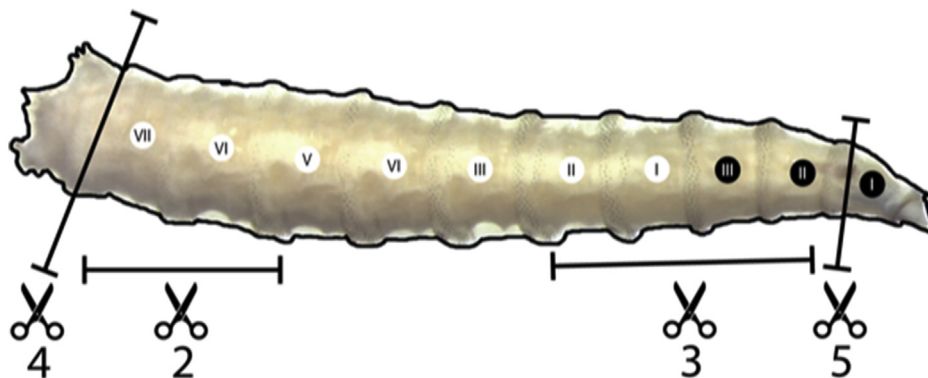**Fig. 4.** Example of a cleared whole larval specimen.

Fig. 5. Dipteran larva illustrating the locations for cuts 2−5. Where thoracic segments are denoted by black circles, and abdominal segments are denoted by white circles.

epistomal plate and intermediate sclerite and they will no longer be usable for identification purposes.

6) Mount the divided cephaloskeleton using a PVA mounting medium (Bioquip, PVA Mounting Medium 6371A) and a cover slip (Bioquip, Square Cover Slips 6341B). CAUTION: it is important to take your time with this step so you can lay out all the structures in a uniform manner. See discussion below for more details. As the mounting medium dries, add weight to the cover slip to keep the parts spread apart (Fig. 6). We found adding a few extra cover slips (anywhere from 2-3 additional cover slips) on top did the job.

3. Results and discussion

This technique was developed from the need to have a standard protocol to prepare muscomorpha larval mouthhooks for analysis using geometric morphometrics. Specimens processed using this method end up being divided and spread neatly on a microscope slide, maintaining all the original characters of interest, apart from those damaged in the process of dissection, i.e., the epistomal plate and intermediate sclerite. Following our clearing and mounting procedure will result in a specimen mounted on a slide with minimal loss of the two-dimensional diagnostic features for the individual anatomical characters, although the spatial relationships between the characters are lost in the process due to the manipulation. Geometric morphometric tools available are able to ignore issues such as rotation, translation, and scaling [22]. This means that as long as both sides of the cephalopharyngeal skeleton are preserved without damage on the slide, the investigator can perform various types of statistical analysis on them (e.g. generalized Procrustes analysis; principal components analysis). We recommend that anyone trying to use shape analysis on these parts,
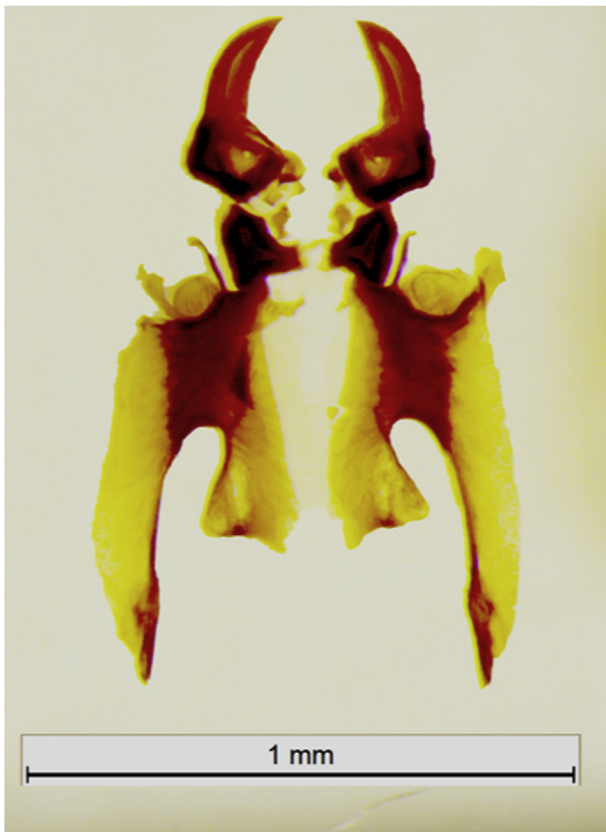
Fig. 6. Divided cephaloskeleton of a third instar *Lucilia sericata* larva with scale for reference.

analyze the mouthhooks separately from the rest of the cephalopharyngeal anatomy. Because mouthhooks and the rest of the skeleton are not connected by a solid structure, but rather by tissue that dissolves during clearing, the mouthhooks and the other sclerites do not maintain the same relative configuration during the clearing and mounting process and thus should not be analyzed together as a single unit. However, this is true even if the specimens were retained *in situ*, since there are points of articulation on the cephalopharyngeal anatomy that do not ensure the same relative positioning across specimens or species. Because of this, geometric

morphometric analysis should be portioned for each individually articulating structure within the cephalopharyngeal anatomy.

This standard protocol for preparing specimens, allows multiple questions to be asked of the same specimen. Even though the protocol was developed to facilitate specimen identification for forensic entomology, geometric morphometrics is able to provide data that can help answer questions concerning phylogenetics [23], evolution [24], and feeding behavior [25,26] as well. Once the specimen is preserved as described, all that is needed for analysis is an image of the mounted specimen with a graticule for scale. Using this standard protocol also simplifies the ability to share specimens with collaborators, since each collaborator can prepare specimens from their regions and then share the images.

We have limited our work to third instar specimens at this time, since they are the largest specimens and the easiest to work with. Expansion of this work to first and second instar muscomorpha specimens if entirely possible, although we think care should be taken to determine the best clearing times so as to not damage the cephalopharyngeal skeletons of such specimens. Tackling first and second instars would be a good expansion of our work.

Although this method is destructive to the specimen, key features used for identification are preserved. Notably, the skin attachment sites for the cuticular layer, also proposed for species identification [27,28], could be laid out on the same slide; as could the caudal end of the specimen, preserving the posterior spiracles under cover slips. Posterior spiracles are another element that is important for species identification that can be preserved on the same slide. We provide an example of how that might look with Fig. 7.

Even if geometric morphometrics is not the end goal, the clearing technique provides a procedure that aids larval identification even without dissection. The potassium hydroxide solution clears the specimen's cuticular layer and disintegrates the fatty tissue that normally obstructs the view. Once the specimen is more transparent, the important diagnostic features (e.g. − mouthhooks, anterior and posterior spiracles) can be viewed without dissection (Fig. 4). The dissection process we describe is delicate and requires practice to perfect, thus results varied between our first samples and those produced after some experience (Fig. 8a−d). We recommend users learn on disposable specimens before beginning the work with research or casework samples.

We developed this protocol to provide a standard for shape generation for scientists utilizing geometric morphometrics on larval Diptera. Shape analysis is conducted on images of these
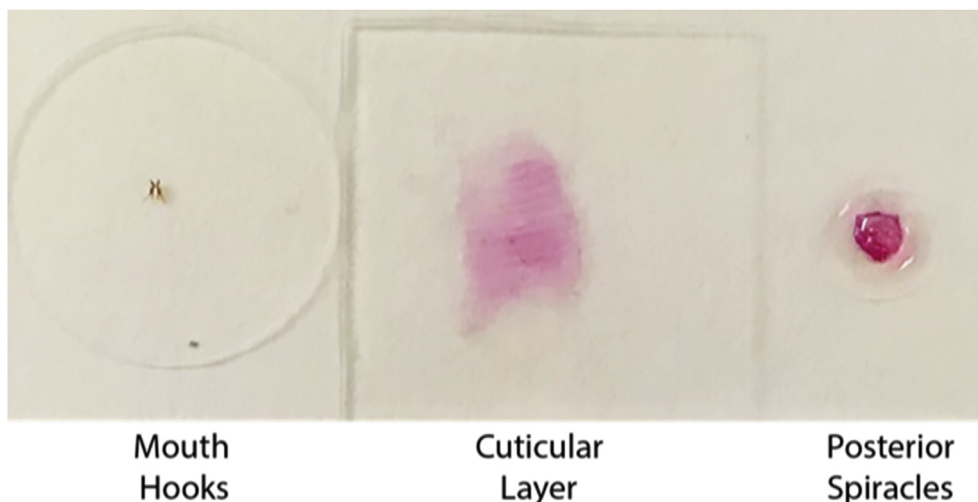

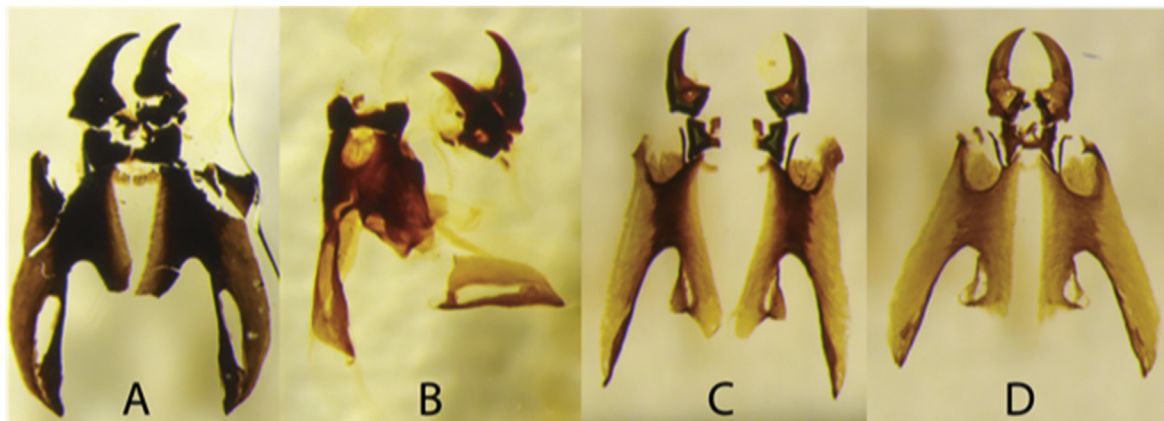**Fig. 7.** Slide with the mouth hooks, the abdominal cuticle, and the posterior spiracle preserved.

Fig. 8. Examples of whole and damaged cephaloskeleton specimens. A and B are examples of *Sarcophaga bullata* samples damaged during dissection that are not fit for analysis. C and D are examples of *Lucilia sericata* and *Phormia regina* third instar specimens that are fit for analysis.

cephalopharyngeal skeletons, so any deviation between specimens due to deviation from a strict protocol limits the possibility for comparison with other specimens. Larvae preserved with this technique could be useful for sharing between laboratories as well; images can be uploaded to MorphBank [29] and the sharing of specimen data in this manner would greatly increase the value of such work, moving entomologists towards building cohesive morphological-based world catalogs that are cross-comparable for the first time.

CRediT authorship contribution statement

Gregory Nigoghosian: Conceptualization, Methodology, Validation, Investigation, Data curation, Writing - original draft, Visualization, Supervision, Project administration, Funding acquisition. **Lauren M. Weidner:** Methodology, Investigation, Writing - review & editing, Funding acquisition. **Trevor I. Stamper:** Conceptualization, Methodology, Resources, Writing - original draft, Writing - review & editing, review & editing, Visualization, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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