JOURNAL OF CRUSTACEAN BIOLOGY, 30(3): 351-356, 2010

PHOTOGRAPHY OF SOFT-BODIED CRUSTACEANS VIA DRYING, WHITENING, AND SPLICING

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ABSTRACT

It is difficult to take good detailed pictures of soft-bodied crustaceans (and other invertebrates); photographing animals in alcohol leads to problems with lighting and focus while drying out soft-bodied animals for photography distorts their morphology. To help better capture surface detail, the following procedures are proposed. Specimens should be fixed and stained with a general tissue stain in order to ensure an even coloration, followed by either chemical drying (using hexamethyldisilazane) or critical point drying. Specimens are then mounted to allow for whitening with either ammonium chloride (NH_4Cl) or magnesium oxide (MgO) to highlight areas of high topographic relief. Lastly, to increase the depth of field in the resulting image, photographs should be taken at sequential planes of focus and then spliced together using a software package for that purpose (i.e., Helicon Focus or Leica Applications Suite). This method is especially useful for showing surface detail and for making comparative images of modern crustaceans to compare with fossil specimens.

KEY WORDS: photography, techniques

DOI: 10.1651/09-3253.1

INTRODUCTION

Advances in imaging technology have given us a wealth of new ways to photograph and document crustacean morphology, e.g., digital camera, scanning electron microscopy, micro-computed tomography, confocal microscopy, etc. However, certain operational problems remain. Scanning electron microscopes (SEMs) provide images of amazing detail and resolution, but only small areas (or small crustaceans) can be photographed (making whole-body images of larger crustaceans is usually impossible except via time-consuming mosaic images). Photography (either microscopic or macroscopic) of crustaceans preserved in alcohol presents a host of challenges from lighting to depth of field (see Fig. 1B). Furthermore, coloration of the specimen (often rendered meaningless by preservatives) can obscure the topographic detail of the specimen. As a result, illustrations of crustacean morphology have largely depended on drawings, which themselves possess problems related to subjectivity, skill, and artistic style.

This note describes a procedure for photographing small lightly-sclerotized crustaceans. It does not involve new methods, but rather it is a hybrid of two well-established techniques: a biological technique of critical point drying or desiccation via hexamethyldisilazane (HMDS), and a paleontological technique (specimen whitening). The drying of the specimen eliminates the difficulty of posing and photographing a specimen in alcohol, while the whitening of the specimen highlights the topographic relief of the specimen. This method is applicable to other invertebrate taxa, and elements of the method (whitening) can be easily applied to larger, more heavily mineralized crustaceans. When coupled with software that can splice together photographs taken at different focal planes of the specimen, the method yields sharp images of very high quality and clarity. Images produced are comparable to those produced by SEM but can be captured with a typical microscope camera or camera stand.

MATERIALS AND METHODS

Crustacean specimens used in this study are housed in the Yale Peabody Museum Invertebrate Zoology collection (YPM IZ). Specimens of *Triops longicaudata* [YPM IZ.47917; Branchiopoda: Notostraca] were hatched by the author in the lab, and those of *Lepidurus* sp. [YPM IZ.43110; Branchiopoda: Notostraca] from Tehama County, California, were taken from the YPM Invertebrate Zoology collections. Specimens of *Armadillidium vulgare* [YPM IZ.47923] and *Haplophiloscia vittata* [YPM IZ.47922; Malacostraca: Peracarida: Isopoda] were collected by the author from a shower stall in Fort Getty Park, Jamestown, Rhode Island in July, 2008. Specimens of the malacostracan taxa *Crangon septemspinosa* [YPM IZ.47919 (air dried), YPM IZ.47920 (HMDS dried) and YPM IZ.47925 (ethanol preserved); Decapoda: Pleocyemata: Caridea] and *Leptocheirus pinguis* [YPM IZ.47918; Peracarida: Amphipoda: Gammaridea] were collected by the author during the August 2009 Bioblitz conducted by the Yale Peabody Museum of Natural History and Connecticut's Beardsley Zoo in Long Island Sound. All specimens except for *Lepidurus* sp. were stained with hematoxylin. *Lepidurus* sp. was stained with black ink. Images were collected on a Leica camera and microscope and then processed in Helicon Focus and Photoshop.

PROCEDURE

The procedure generally includes the following steps (in order): preservation/staining, drying, mounting, whitening, and splicing together of photographs of different focal lengths. This sequence can vary slightly depending on the initial condition and size of the specimens, and these variations will be noted in the discussion of each step below. This method must be considered destructive, but destructive in the same sense that standard SEM preparation is destructive; proper curation in a silica gel desiccator will ensure their utility well into the future.

Preservation/Staining.—There are many methods for preserving biological specimens (Galigher and Kozloff, 1971); these methods vary depending on the target tissue and/or the type of preservation desired. For this type of photography, a method of preservation that fixes and stiffens soft-tissue is highly desirable. The author has found

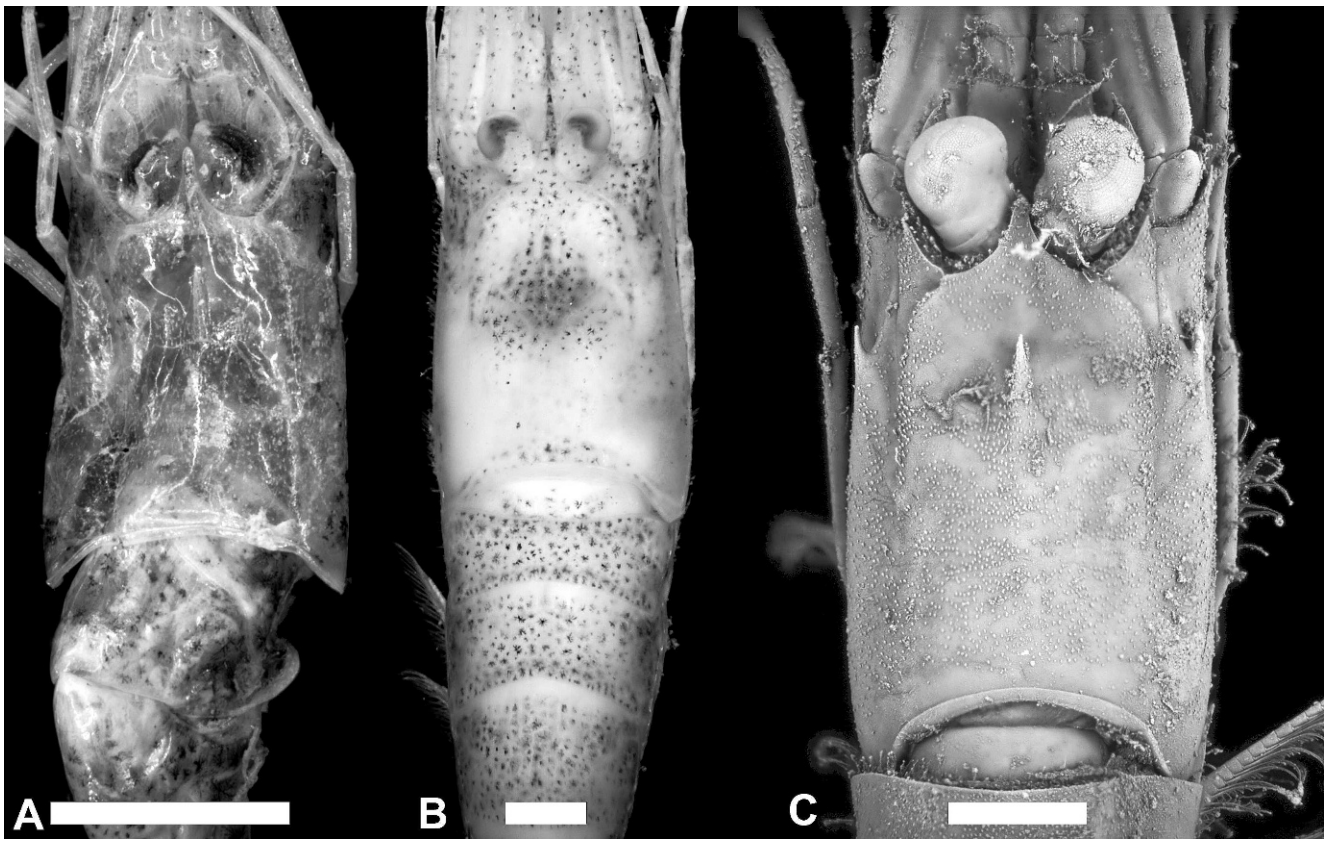

Fig. 1. Comparison of the ''photogenic'' quality of specimens belonging to *Crangon septemspinosa* that have been. A, dried in air following preservation in ethanol (YPM IZ.47919; image composed of 13 slices); B, photographed in ethanol (YPM IZ.47925; image composed of 18 slices); and C, stained, dried and whitened following the procedure described herein (YPM IZ.47920; image composed of 17 slices). Note the detail of the carapace shown in image C. All scale bars are 1 mm.

Felgenhauer's (1987) techniques of specimen preparation for SEM very useful (using gluteraldehyde and osmium tetroxide), but the choice of preservative will vary by taxon and personal preference.

Color patterns can be a key characteristic in taxonomy. In life, the patterns can help to camouflage the animal; in death, those same patterns can obscure and mask the surface detail of the animal. For this reason, it is often helpful to stain the animal with a tissue stain. The goal of this step is to try to make the animals a more uniform hue and to mask residual color patterns so that the whitening procedure (below) will give better results. A useful side effect of Felgenhauer's (1987) specimen preparation protocol is that the use of osmium tetroxide often turns the animals a dark brown. The author has had good luck using hematoxylin to stain the specimens an even pink (Rose Bengal should work as well). Another method that has been used in crustaceans utilizes silver nitrate (see Cummings, 1956; Green, 2001, for its use on microfossils), but this method relies on chemicals, i.e., photographic developer, that are becoming increasingly difficult to obtain due to the prevalence of digital photography. Specimens that have been stored in alcohol for long periods of time typically become lighter in color, and the usefulness of these specimens, in particular, is helped by staining so that they contrast with the whitening agent. Any heavily mineralized specimens that are already dry can be essentially painted with thin India ink. Consult Galigher and Kozloff (1971) for additional information concerning biological stains.

Drying.—Typically, drying soft, lightly sclerotized specimens ruins them due to the surface tension of the storage solution. Desiccation causes them to collapse, shrink,

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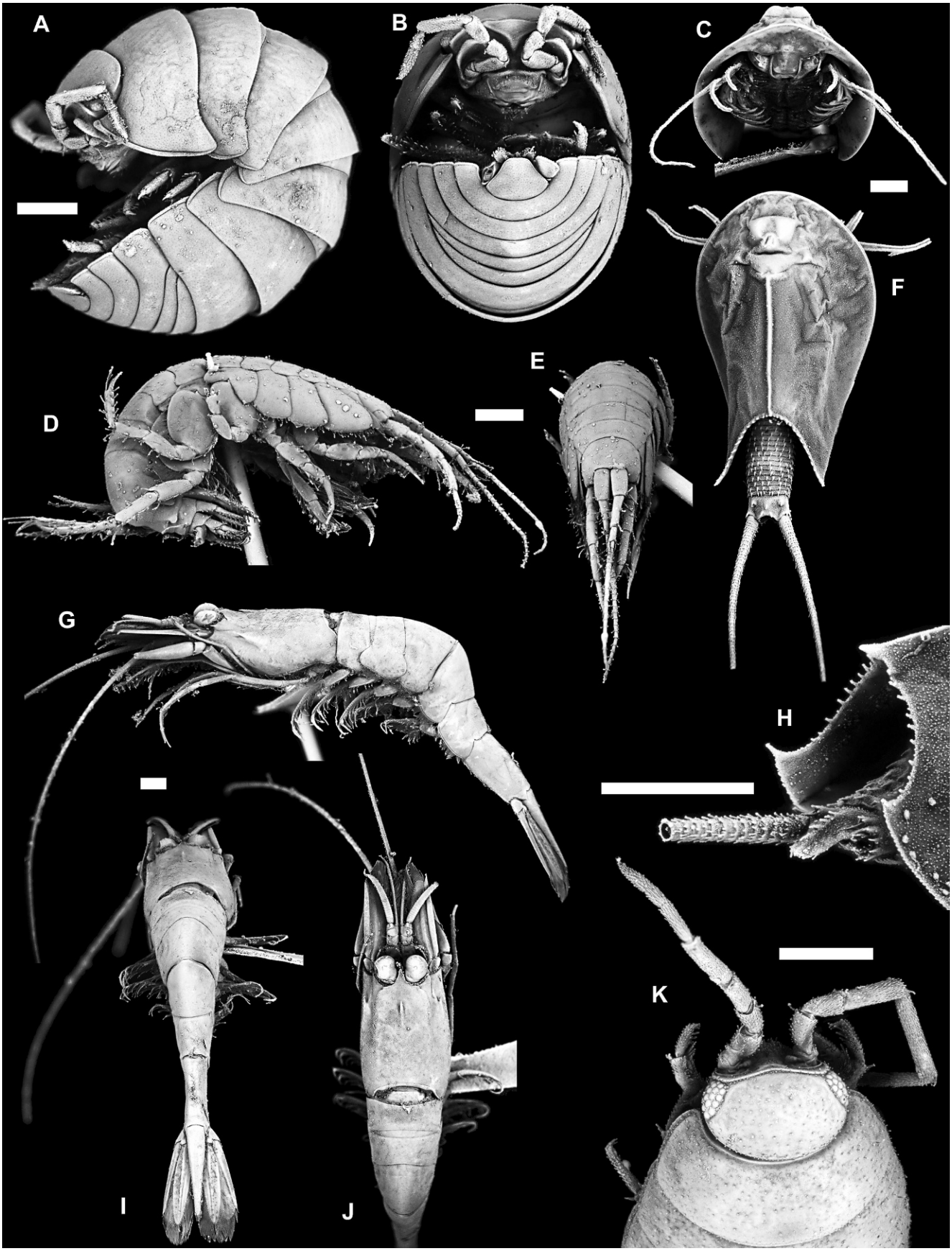
Fig. 2. Images of crustaceans taken using the procedure described herein. A and B, partially enrolled specimen of *Armadillidium vulgare* (YPM IZ.47923) shown in left lateral (image composed of 2 slices) and anterior views (image composed of 5 slices). The scale bar for both is next to A. C and F, *Triops longicaudata* (YPM IZ.47917) shown in anterior (image composed of 3 slices) and dorsal views (image composed of 3 slices), with the scale bar in between. D and E, *Leptocheirus pinguis* (YPM IZ.47918) in tilted postero-right lateral (image composed of 10 slices) and anterior views (image composed of 11 slices), with the scale bar in between. G, I and J, *Crangon septemspinosa* (YPM IZ.47920) shown in right lateral (image composed of 3 slices), posterior (image composed of 4 slices) and dorsal views (image composed of 1 slice), with the scale bar in between. H, *Lepidurus* sp. (YPM IZ.043110) in an oblique posterior view (image composed of 10 slices), with the scale bar above the left caudal furca. K, the head of *Haplophiloscia vittata* (YPM IZ.47922) in dorsal view (image composed of 12 slices), with the scale bar above. All scale bars are 1 mm.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shrivel, and to become distorted (Fig. 1A). However, the whitening procedure (below) only works on dry specimens. To avoid the ill effects of desiccation, one of two methods can be employed: critical point drying or treatment with HMDS. Critical point drying (CPD) is a standard technique used to prepare microscopic specimens for SEM analysis (Bray, 2000). During CPD, a specimen is put into liquid CO_2 under pressure, and the temperature is raised until the critical point is reached where the CO_2 turns into the gaseous phase (Bray, 2000). The small size of the chambers of most CPD units may present a problem for drying larger specimens. Treatment with hexamethydisilazane (HMDS; see Moraes and Bouzon, 1995; Nation, 1983; Heraty and Hawks, 1998) is another method of drying specimens (all of the images here were taken specimens dried via HMDS). The precise way in which HMDS works is still unknown (Rumph and Turner, 1998), but Nation suggested that it might form silyl ethers within tissues and that those tissues may be stiffened by protein cross-links (Nation, 1983; Rumph and Turner, 1998). Several studies have examined the comparative effectiveness of CPD and HMDS on arthropods, and have found both to produce good results (Heraty and Hawks, 1998; Laforsch and Tollrian, 2000; Meyer and Melzer, 2004; Moraes and Bouson, 1995; Rumph and Turner, 1998), with HMDS treatment being considerably less expensive. In theory, both methods could probably be reversed so that the specimens could be returned to alcohol for storage; no one has yet devised a method to do so.

Mounting.—The way in which a specimen is mounted for photography is highly dependant on the specimen's size, weight, and shape, as well as the area of interest. Mounting a specimen on a pin, for example, allows for easy rotation and positioning into different orientations (especially when aided by a small block of clay). The best results for whole-specimen photography will come when the specimen is elevated and photographed above a black background. The reasons for this are twofold: elevation of the specimen makes whitening all parts of a specimen easier and it puts the background out of focus (an out-of-focus black background makes the image composition cleaner and is easily removed in Photoshop). Careful framing of the photograph can allow for easy removal of the pin from image during image processing (especially if the pin is black in color). However, one should never digitally remove a mounting medium (or any other object/blemish) from an image if doing so would make the image deceptive misleading (McInnes, 2001; compare Fig. 2C with Fig. 2F).

Entomologists have long used elevated mounts for specimens; entomological manuals contain many helpful tips (Gibb and Oseto, 2006). Mounting specimens is a trade-off between the quality of the resulting images, and the loss of morphology that necessarily occurs during mounting. This loss of morphology can be minimized by choosing a suitable mounting method and applying the mount onto the specimen in an area of minimal interest. Larger specimens can be positioned with a bit of clay, but the oils in the clay will interfere with whitening in that area. Mounting specimens on pin is quick and easy. However, it does usually require puncturing the animal, which obviously damages the external morphology. Small animals can be difficult to spear precisely, and an errant pinpoint (see Fig. 2D, E) can destroy a specimen. Larger specimens can usually accommodate a pin in an inconspicuous place, like the anal opening. Specimens can also be glued to a pin for the same effect (larger items like toothpicks and dowels work equally well for larger specimens). Entomologists frequently glue small specimens on tiny paper triangles called 'points' ($\approx$ 5-10mm) that are then speared with a pin, and this method can be employed in the context of this paper (see Fig. 2C, F, G, I, J). It is recommended that one uses points made out of black paper rather than the entomology points made out of white paper so that they can be more easily relegated to the background in the final photograph. Water-based glues should likely be avoided for use with dried specimens to avoid causing local collapse of morphological structures. It is unlikely that one could employ a water-soluble glue, e.g., gum tragacanth, without damaging the specimen during the application of the relevant solvent.

Whitening.—Different methods of whitening all essentially work the same way; they apply a fine-grained compound (in the form of a sublimate vapor or smoke) to a specimen to highlight the areas of high topographic relief (Feldmann, 1989; Green, 2001). The particles of the compound will tend to land on high areas, thereby emphasizing areas of high relief as white in color. This helps to ''bring out'' details of sculpture/ornamentation or the shape of furrows, sutures, etc. Two main whitening compounds, each using a different method for their application, have been employed in paleontology: ammonium chloride, and magnesium oxide. Each compound has its own advantages and disadvantages. Both methods are potentially hazardous to human health, so safety protocols should be observed. Whitening should always be done under a fume hood (Feldmann, 1989; Green, 2001).

Ammonium chloride (NH_4Cl) is perhaps the more common of the two methods (it is the method employed on the specimens illustrated herein). It is relatively safe and easy to aim and apply to the specimen. The procedure for its application has been detailed and thoroughly referenced by Green (2001). In brief, it involves heating ammonium chloride powder over a bunsen burner in a modified glass drying tube. When the ammonium chloride begins to sublimate (turn to vapor within the drying tube), the vapors are 'poofed' on the specimen by pumping a rubber atomizer bulb attached to one end of the drying tube. The sublimate is applied evenly from all directions, with care taken to avoid overwhitening (with some practice and experience, one will develop a feeling for how to recognize and avoid overwhitening). Once finished, the specimen should be photographied quickly. The coating is very susceptible to humidity in the air and will begin to rehydrate, fade, and crystallize into coarser grains when water or water vapor is present. Whitening and photography should be conducted in an environment of low humidity or a humidity-controlled room. After whitening, care should be taken to avoid handling or breathing on the specimens, as the moisture from those sources will remove the coating. Ammonium chloride can form HCl when in contact with water vapor.

Fossil specimens are normally rinsed in water to remove the coating (and any chance of etching by HCl), but dried biological specimens cannot be treated in quite the same way. Instead, it is herein recommended that coated specimens are kept in desiccator with silica gel in order to preserve the specimen. This is a precautionary measure; the amounts of HCl produced seem to be very small and any damage would be at a very fine level. In the author's experience, inadvertent rehydration of the ammonium chloride sublimate into HCl produces no noticeable macroscopic damage (despite such ill-advised actions as breathing on calcareous specimens to remove the whitening).

Use of magnesium oxide (MgO) is another way to whiten specimens. Its use avoids the potentially corrosive effects of ammonium chloride, but it is more difficult to apply (Green, 2001). To apply the coating, a specimen is rapidly moved back and forth through the fumes from a burning piece of magnesium ribbon, with the motion ensuring and even coating (Green, 2001). Use of magnesium oxide has several benefits over ammonium chloride: it generates a finer-grained sublimate that is insoluble in water and does not generate corrosive HCl. However, it is much more difficult to apply, especially to larger specimens. Proximity to the burning magnesium oxide may damage dried biological material, and fragile specimens can be damaged or shaken free of their mounting by rapid movement through the magnesium oxide fumes.

Both methods outlined above require some amount of practice for the best results. They have been employed successfully on fossil specimens as small as a one millimeter across. The whitening can also be readily removed from the pin, point, or toothpick on which the specimen in mounted either by a fine paintbrush dipped in water (ammonium chloride), or by brushing it off with a dry fine paintbrush (magnesium oxide). In this way, the mounting media can be more easily kept from prominence in the final image. However, one drawback of whitening is that ammonium chloride may make small spines look blunted and setae appear to have globose tips due to the greater adherence of the sublimate to those raised structures (presumably MgO would have the same effect, though perhaps to a lesser degree due to its finer grain size).

Image Splicing.—A perennial problem of photography, and microscopic photography in particular, is the depth of field. For most specimens, it is impossible to have the complete specimen in focus in one image. However, several companies have recently developed software to cope with this problem. The software packages work by means of digitally combining a ''stack'' of images taken at different focal depths into one image by combining the focused areas. Leica Microsystems produces a software package called Leica Application Suite that works in conjunction with their automated stage microscope. The advantage to it is that you can specify the precise interval at which you want the camera to take the pictures, and the camera will adjust the focus automatically. Helicon Focus (produced by Heliconsoft) is another software package that can do the same thing with micro and macro images (the author notes that he used a Leica automated stage microscope manually, and processed the image stack in Helicon Focus). These software packages produce great images, but occasionally they generate image artifacts—bands of blurriness (due to having to great of a vertical distance between the focal planes), halos around objects, and outline duplication. This last artifact is the most dangerous to the image quality; the final image should be checked against the images in the image stack for any artifacts.

Any image produced in this way should be noted as such, as with any digital modification. The line between image processing, image enhancement, and outright fakery can be blurry (McInnes, 2001), and the defense against its abuse is honesty. In this paper, a mounting point was digitally removed from Fig. 2A, B as the smooth contours of the isopod's exoskeleton provided a ready contrast to the point. However, mounting points were not removed from other images (i.e., Fig. 2C, G, I, J) either because the boundaries of the point were not clearly defined from the morphology, or obscured some portion of the morphology, or because removing it would have in some way yielded a deceptive image.

Conclusion

The original impetus for this project was to create a way to take comparable photos of both fossil and modern notostracan carapaces. In this way, the combination of biological and paleontological techniques was a natural outgrowth of comparative morphological research. It is hoped that the methodological cross-pollination that spawned this procedure will likewise lead to an intellectual cross-pollination between morphological research in neontology and paleontology. Morphological drawings will always carry some level of subjectivity with them; carcinology as a whole will benefit from the addition of clear, detailed, morphological photos to supplement drawings in systematic and comparative research.

Acknowledgements

E. A. Lazo-Wasem provided the initial encouragement to the author to pursue this project and advice at all stages. L. Gall provided advice on entomological approaches to specimen-mounting. The Briggs Lab Group provided helpful discussions. F. R. Schram provided helpful comments on the manuscript. The August 2009 Bioblitz conducted by the Yale Peabody Museum of Natural History and Connecticut's Beardsley Zoo provided an opportunity to collect additional specimens. Lastly, the author wishes to thank his wife, Megan, for allowing collection of the isopods from a campground restroom while on vacation.

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RECEIVED: 11 November 2009.
ACCEPTED: 16 November 2009.