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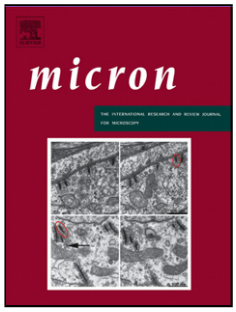

Automontage Microscopy and SEM: A Combined Approach for Documenting Ancient Lice

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PII: S0968-4328(20)30180-3

DOI: https://doi.org/10.1016/j.micron.2020.102931

Reference: JMIC 102931

To appear in: *Micron*

Received Date: 7 February 2020

Revised Date: 19 August 2020

Accepted Date: 20 August 2020

Please cite this article as: Reinhard KJ, de Araújo EP, Searcey NA, Buikstra J, Morrow JJ, Automontage Microscopy and SEM: A Combined Approach for Documenting Ancient Lice, *Micron* (2020), doi: https://doi.org/10.1016/j.micron.2020.102931

Automontage Microscopy and SEM: A Combined Approach for Documenting Ancient Lice

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Highlights

- Archaeological louse specimens are unique and require detailed documentation
- Typically, Scanning Electron Microscopy is applied to lice from mummies
- Automontage provides a nondestructive method to image details of specimens, especially color and internal structures
- We show the value of applying SEM and automontage to the same specimens from mummies

ABSTRACT

Human ectoparasites, including lice, have been recovered from a wide range of archaeological materials. The human head louse, *Pediculus humanus capitis,* has been identified from mummies and sediments for decades. Louse eggs are the body part most commonly encountered and therefore the most frequently quantified. Typically, several types of microscopy are applied for egg documentation. For studies in which quantification of infestation is a goal, counting is done with the naked eye or with the aid of handheld lenses. For determination and stage classification, stereomicroscopy is commonly used. For more detailed examination of microstructure, light microscopy, scanning electron microscopy (SEM), and confocal laser scanning microscopy (CLSM) can be employed. In most reports, researchers use

two or more techniques to accomplish interrelated goals. Automontage microscopy is used to document prehistoric arthropods with good success. Herein, we report the results of a combination of SEM and automontage microscopy to document lice and eggs recovered from South American mummies. This combined approach allows for simultaneous examination of internal and external characteristics. Thirty automontage composite images of 2 adult lice and 16 eggs showed that egg internal morphologies were easily examined showing the within-egg anatomy of emergent nymphs. SEM imaging of 9 lice and 129 eggs was completed. In the case of two adults and several eggs, SEM imaging was accomplish after automontage image capture of the same specimens. This one-to-one image comparison of SEM and automontage shows that transmitted light of automontage reveals egg internal structures and details of the adult lice. SEM allows for high magnification examination of egg, nymph and adult microstructures. We conclude that automontage imaging followed by SEM results in efficient graphic documentation of rare louse specimens.

KEYWORDS: Lice, Automontage Microscopy, Mummy, Nit, *Pediculus humanus*, Scanning Electron Microscopy

1. INTRODUCTION

For studies of head louse infestation (*Pediculus humanus capitis*) from mummy collections, imaging is essential for establishing diagnosis, illustrating the intensity of infestation and documenting the condition of the recovered lice. Mummies are increasingly a limited resource and optimal microscope methods should be standardized for recovery of all these needs from single specimens. In addition, mummies and associated arthropods are of public interest. It is noteworthy that most popular articles written about mummy louse research do not include images of lice from researchers. Instead, journalists select more sensational images of modern lice (Netting, 2000; Abrahams, 2013; Roberts 2019). Apparently, journalists do not consider images presented in peer-reviewed articles sufficiently engaging. Therefore, we are attempting to develop combinations of microscope methods that are both objective for scientific presentation and illustrative for public interest. Thus, we have explored microscopy methods that are useful for diagnosis and also for illustrating prehistoric infestation problems for our science-focused society. In this paper we present a combination of two methods, scanning electron microscopy (SEM) and automontage microscopy that can be used in a complementary fashion to define the biological details of lice while capturing engaging images for popular publications. These methods resolve internal and external specimen details. Automontage microscopy documents internal structure and natural coloration. SEM allows for high magnification view of mite features. Importantly, both methods can be applied to the same specimens.

In previous studies, researchers have focused on quantification of louse infestations of mummies to assess infestation intensity and epidemiology within specific populations (Arriaza and 2012, 2013a; Reinhard and Buikstra, 2003). Typically, this is done by counting lice on the scalps of mummies with the unaided eye or using hand lenses (Figure 1). Detailed methods for

this type of analysis are presented elsewhere (Arriaza et al., 2013a; Reinhard and Buikstra, 2003). By mapping louse discoveries, researchers provided insight into geography and time depth of louse distribution (Araújo et al., 2000; Dittmar, 2000; Dutra et al., 2014; Forbes et al., 2013; Fornaciari et al., 2009; Horne, 1979; Mumcuoglu and Hadas, 2011; Mumcuoglu et al., 2003; Mumcuoglu, and Zias, 1988; Rick et al., 2002; Zias and Mumcuoglu, 1991). For these studies, stereomicroscopy was used to sort lice from archaeological source material such as textiles, sediment (archaeological soils), and scalp/hair samples. High magnification is often employed to examine the microstructure of the eggs, embryos within eggs, nymphs, and adults collected from these contexts. Methods for recovery and cleaning of ectoparasites, including lice, from guinea pig mummies have been presented by Dittmar (2000). For human mummies as well, the separation of dirt, sand, and adhesive decomposition products is an analytical challenge. A literature review that includes methods associated with stated research goals is presented in Table 1.

It is noteworthy that although automontage imaging was applied to arthropods from various archaeological sites with excellent success (Fugassa et al., 2011; Johnson et al., 2008; Morrow et al., 2015, 2016, 2017), it has not been applied to *P. h. capitis* specimens as shown in the literature review. Using the method described in this paper, our lab imaged several types of arthropods. These studies include two separate treatments of prehistoric fragmentary ticks, *Dermacentor andersoni* (Fugassa et al., 2011; Johnson et al., 2008). Diagnosis was obtained by imaging of the dorsal and ventral surfaces of the ticks showing details of the festoons, capitulum, dorsal scutum, palpi and white dorsal markings typical of this species. Pseudoscorpions of the family Cheiridiidae recovered from Peruvian burials were imaged and identified (Morrow et al., 2017). In this study, automontage and confocal laser scanning microscopy were applied.

Automontage imaging was more effective at detailing the integuments, abdominal segments, subtriangular carapaces, and cephalothorax eyes. Additional studies of fly puparia used automontage to image the spiracles allowing identification of the *Hydrotaea capensis* infestation of Medici embalming jars (Morrow et al., 2016) and *Ophyra capensis* from the body of the Blessed Antonio Patrizi, Monticiano, Italy (Morrow et al., 2015). Considering our lab's proven success with automontage imaging of arthropod remains, we proposed applying this technology to *P. h. capitis* specimens from mummies.

Figure 1. Head louse eggs seen on the scalp of a heavily infested mummy. This view of a mummy scalp shows how visible louse eggs are to the naked eye. Infestations can be easily quantified per square centimeter with the unaided eye or with hand lenses (Reinhard and Buikstra, 2003).

Table 1: Literature review showing microscopy methods applied to *P. h. capitis*. 'Sediments' refers to soil from excavations.

Reference	Method(s)	Goal of Imaging
Araújo et al., 2000	SEM	Illustration of the oldest louse egg on an isolated hair from Paleoamerican cave sediments.
Arriaza et al., 2012	ST, SEM, LVSEM	Assessment of an extreme infestation of an Andean child through quantification and presentation of structure of eggs and adults
Arriaza et al., 2013a	Lens, BFLM, LVSEM	Standardization of imaging methods for illustration of eggs and hatching eggs in a study of social determinants of infestation
Arriaza et al., 2013b	Lens, BFLM, LVSEM	Establishment of methods for imaging cementum , egg morphology, and late stage embryos/nymphs exposed in fractured eggs.
Arriaza et al., 2014	BFLM, LVSEM	Documentation of eggs and a nymph hatching from an egg found in delousing combs.
Dutra et al., 2014	BFLM, LVSEM, ST,	Presentation of an adhesive tape method for fracturing eggs to examine late stage embryos. Also to show structure of eggs, nymphs and adults.
Forbes et al., 2013	ST	Illustration of louse from a prehistoric Inughuit winter house in Greenland discovered in the process of mapping the distribution of louse remains within the house.
Fornaciari et al., 2009	ST, SEM	Documentation of louse fragments and cementum on hair shafts recovered from locks of hair associated with King Ferdinand II of Aragon.
Horne, P., 1979	BFLM, SEM	To develop a method for recovering and preparing lice for SEM analysis from an Aleutian mummy.
Mumcuoglu and Hadas, 2011	ST, BFLM	Examination of a Roman Period delousing comb revealed high magnification details of a nymph leg and head.
Mumcuoglu et	ST,	Presentation of high magnification details of a nymph

al., 2003	LSCM	leg recovered from Masada textiles.
Mumcuoglu, and Zias, 1988	ST	Imaging a nymph and an egg recovered lice from delousing combs ranging in age from first century BC to the eighth century AD.
Rick et al., 2002	ST, BFLM	Diagnosis of *Pthirus pubis* infestations from Atacama mummies featuring louse eggs on pubic hair and an adults from clothing.
Riviera et al., 2008	BFLM	Portrayal of lice recovered from 4,000 year old Atacama mummies.
Raoult et al., 2005	ST, SEM, PCR	To photograph adult lice fragments recovered from sediments as part of a broad multidisciplinary approach to recovering evidence of louse-borne bacterial infections from Napoleonic mass graves.
Zias and Mumcuoglu, 1991	BFLM	Diagnosis of pediculosis based on an egg on an isolated hair from Nahal Hemar Cave sediments, dating between 8920-8320 years ago.

BFLM=bright field light microscopy, SEM= scanning electron microscopy, LVSEM= low vacuum scanning electron microscopy, ST= stereomicroscopy, LSCM = laser scanning confocal microscopy, and Lens = hand lens examination.

Automontage microscopy is a digital image processing technique designed to produce optimized focal depth in stereo light microscopy, creating a 3D reconstruction from a sequence of multi-focused images. The technique results in the stacking of multiple images and optimizes the resolution of the composite images produced via refined stereomicroscopy. Automontage software combines the series of images taken at different depths of field to create a focus stack, which results in a final image that shows the entire object in focus.

2. MATERIALS AND METHODS

2.1 *Specimen Recovery*

Our goal was to compare automontage microscopy and SEM techniques for the imaging of louse egg developmental stages and anatomical features in archaeological specimens. We used specimens collected from hair samples extracted from mummified individuals of the Osmore Valley in Peru who lived between 750-1350 CE at the archaeological sites of El Algodonal and Chiribaya Alta. Radiocarbon dates for the sites are presented by Owen (2015). These samples were collected in Peru in August of 1990. Initial sorting and documentation of infestation of 146 mummies was completed in Peru in a field laboratory (Reinhard and Buikstra, 2003). Herein, 'sorting' refers to the process of separating louse remains from sediment (archaeological soil), hair, textiles with associated dust, and adherent fine particles of sand that infiltrate mummy coverings. Permission to analyze the lice was provided by the Instituto Nacional de Cultura del Perú. The specimens had been archived at the Pathoecology Laboratory in the School of Natural Resources at the University of Nebraska – Lincoln. For this analysis, the louse and egg specimens were collected from material archived at the pathoecology laboratory in the School of Natural Resources at the University of Nebraska – Lincoln.

2.1 *Automontage Imaging*

To image louse eggs in a way that allowed the internal nymph appendages to be visible through the eggs' shells, initially a set of dual gooseneck fiber-optic light sources were used. These light sources produced too much reflectivity from the eggs' shell to visualize the internal structures. Therefore, a five-inch diameter Mylar® drafting film tube was placed around the specimen to create a light diffuser. This reduced the reflectivity and allowed the lights to be positioned in a way that made the internal features visible. The louse and egg specimens were examined at a magnification of 0.8–50x under a Leica stereoscope (10447177) and imaged using a PC-mount JVC digital camera (KY-F75U). The automontage software by Syncroscopy was

then used to create 30 to 40 image stacks that generated 3-D images of the specimens. The imaging platform was elevated by a compressed air cushion to eliminate vibrational interference during imaging, resulting in better resolution and image stitching.

2.2 *SEM Sample Preparation and Imaging*

The desiccated *P. h. capitis* specimens were mounted on SEM specimen stubs and sputter coated with gold palladium. Specimen mounts were notched to create directional markers for orienting to sample position during microscopy. The specimens were examined using a Hitachi S-3000N Variable Pressure Scanning Electron Microscope with the operating voltage set to 25 mV and variable working distance and magnification.

3. RESULTS

3.1 *Automontage Imaging*

The analysis of 2 lice and 16 eggs via automontage resulted in a total of 30 composite images. The images captured with this method are pixel-dense and therefore can be magnified for detailed analysis with program such as Preview and PhotoShop. The internal morphologies were easily examined, revealing the anatomy of emerging nymphs (Figures 2 and 3) and adults (Figure 4). We observed arrested emergence in 8 eggs (Figures 2 and 3). A single specimen was fragmented, leaving only a portion of the egg along with cementum. Cementum is a adhesive secretion produced by the female to affix the eggs to hair shafts.

One of the eggs was empty with the operculum missing and the nymph having already emerged. Eggs were found in empty hatched states as well as unhatched states with intact opercula. In some cases, the eggs exhibited arrested hatching. This means that the nymphs were in the process of hatching at death. Egg maturation stage was assessed for each egg specimen. In

total, 7 eggs were determined to be in the early embryonic stage. None of the embryos within the eggs were observed to be in the intermediate or the late stage of development.

3.2 *SEM Imaging*

The analysis of 9 lice and 129 eggs via SEM resulted in a total of 148 images (Figures 2 and 3). SEM could not resolve the internal structures. In total, 42 eggs were imaged with intact opercula; 42 eggs were recovered mostly intact without opercula, and 7 eggs were in arrested emergence. Also, 28 fragmented eggs and 10 areas of residual cementum indicating previous egg presence were imaged.

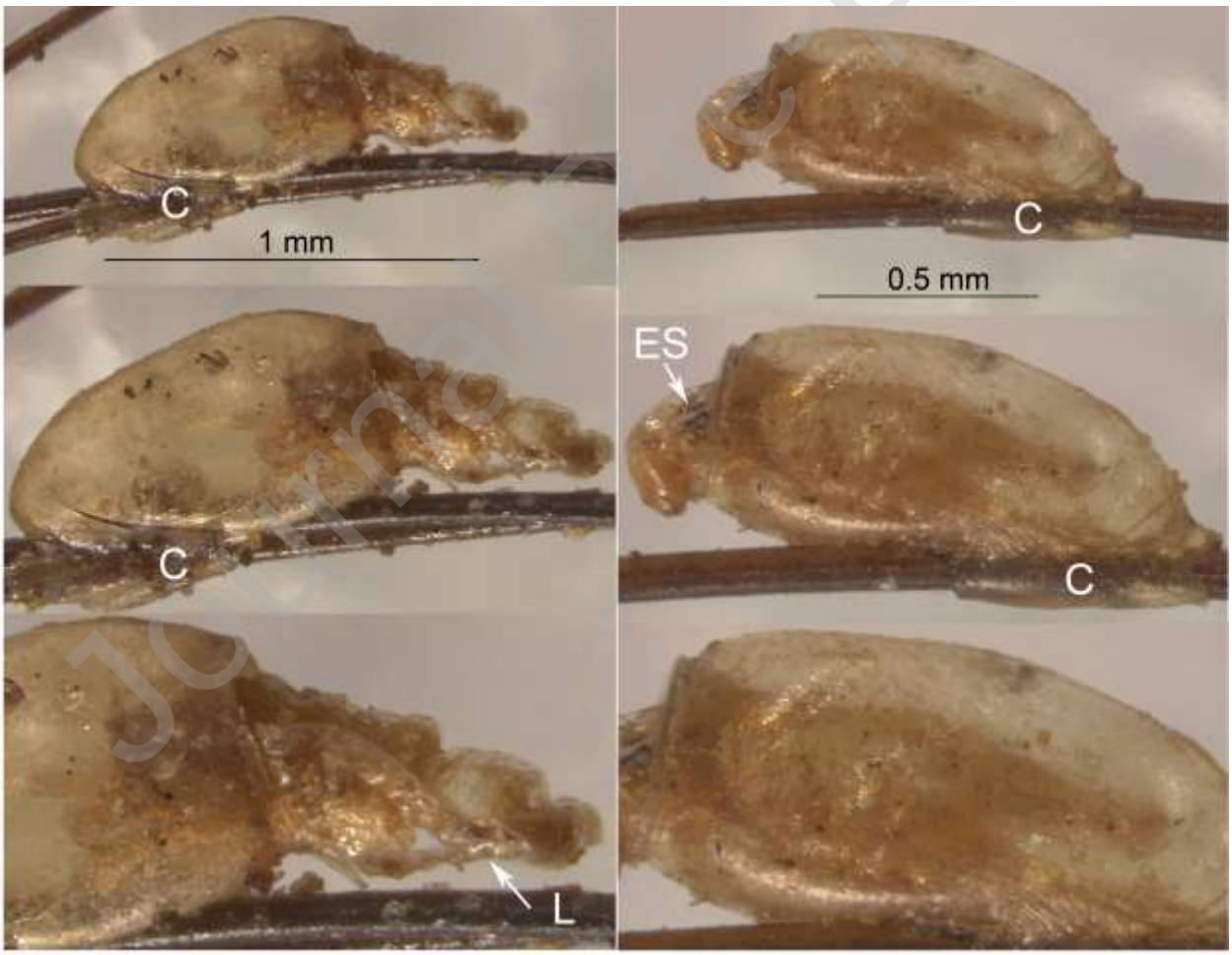

Figure 2. These images show details of two hatching *P. h. capitis*. eggs visible with automontage microscopy including a leg (L) protruding from an egg. The ability to transmit diagonal light through the eggs shows internal nymph bodies and legs. We can also observe the cementum (C) used to attach eggs hair and the eye spot (ES).

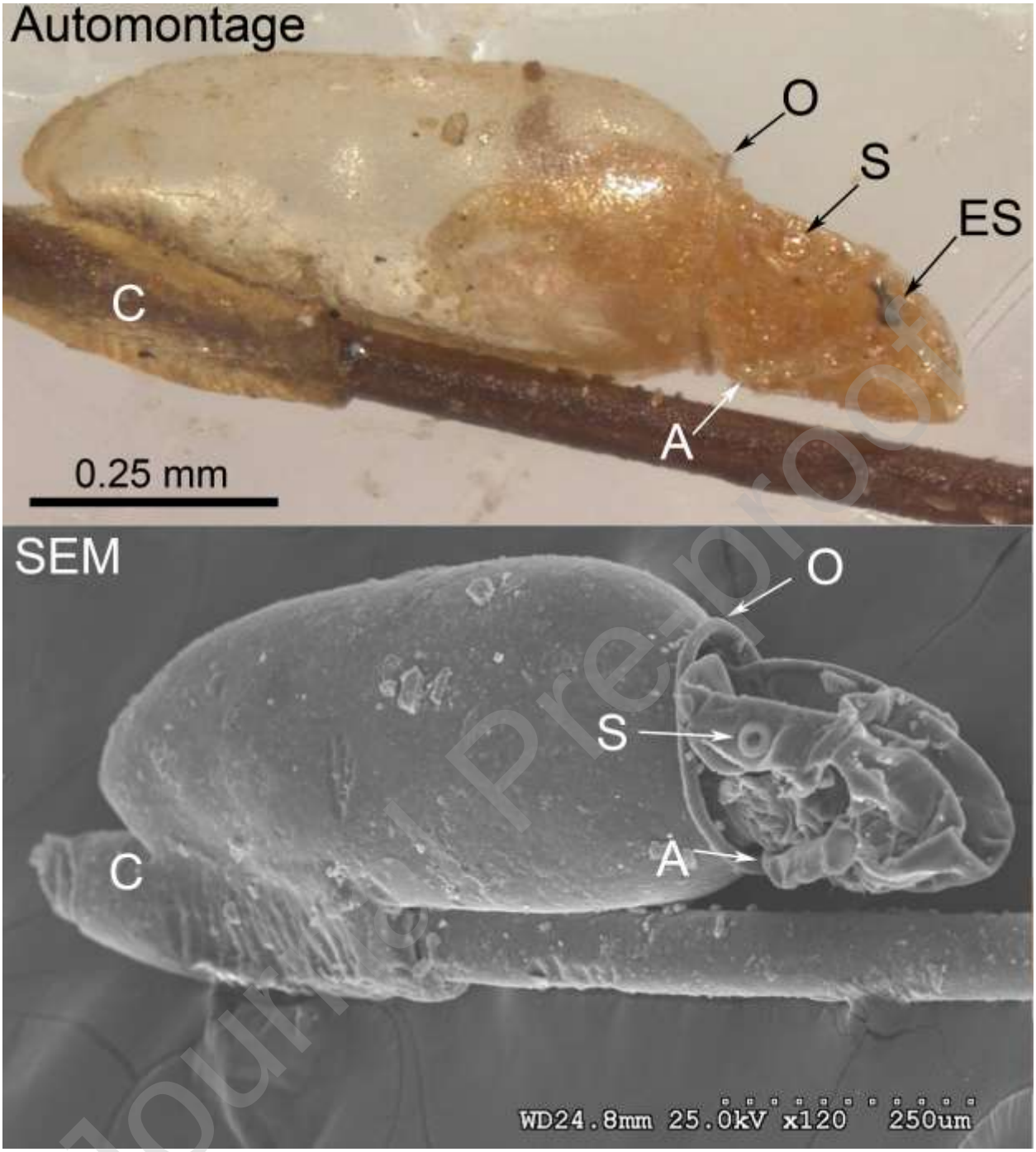

Figure 3. Comparison of automontage and SEM of the same specimen of an emergent louse nymph. In the automontage image, the posterior appendages within the egg are visible and the eye spot (ES) is apparent on the head. The SEM image resolves details of the anterior appendages. In both images we can see the presence of one spiracle (S), more evident in the

SEM image and possibly an antenna (A) below. In the SEM image the details of the opercular rim (O) are more apparent when compared to the automontage image. Cementum (C) is visible in both images.

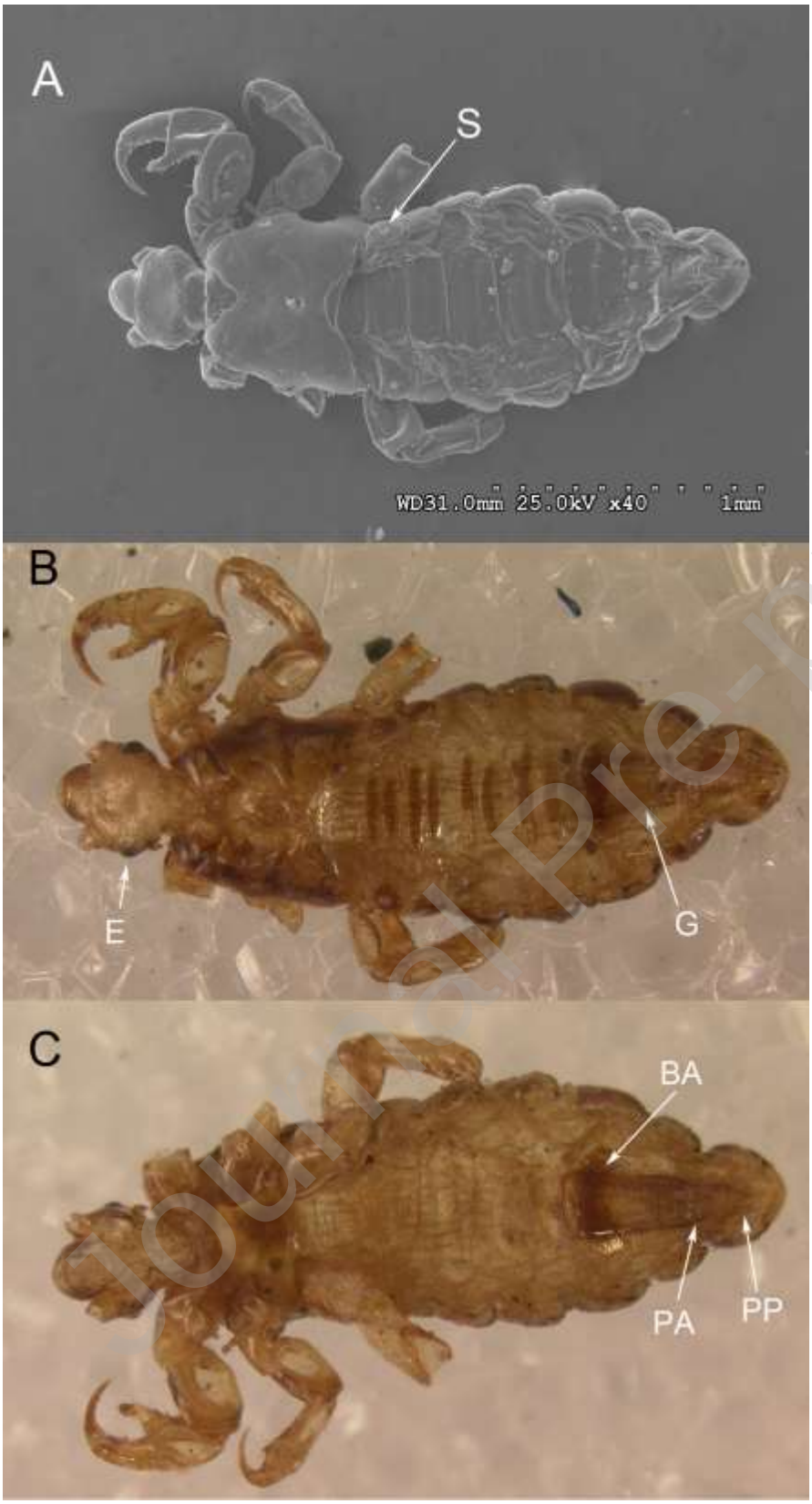

Figure 4. Automontage and SEM comparison of a fragmentary *P. h. capitis*. A and B show the dorsal sides. C shows the ventral side. Spiracles (S), the details of the head, thorax and abdomen are observable in the SEM image. Automontage is effective at capturing the details of coloration and internal morphology (BandC). The eyes (E), abdominal paratergal plates, and thickness variations of the thoracic exoskeleton are evident. Also, the louse male genitalia (G) are visible including basal apodeme (BA), paramere (PA) and pseudopenis (PP; in ventral view).

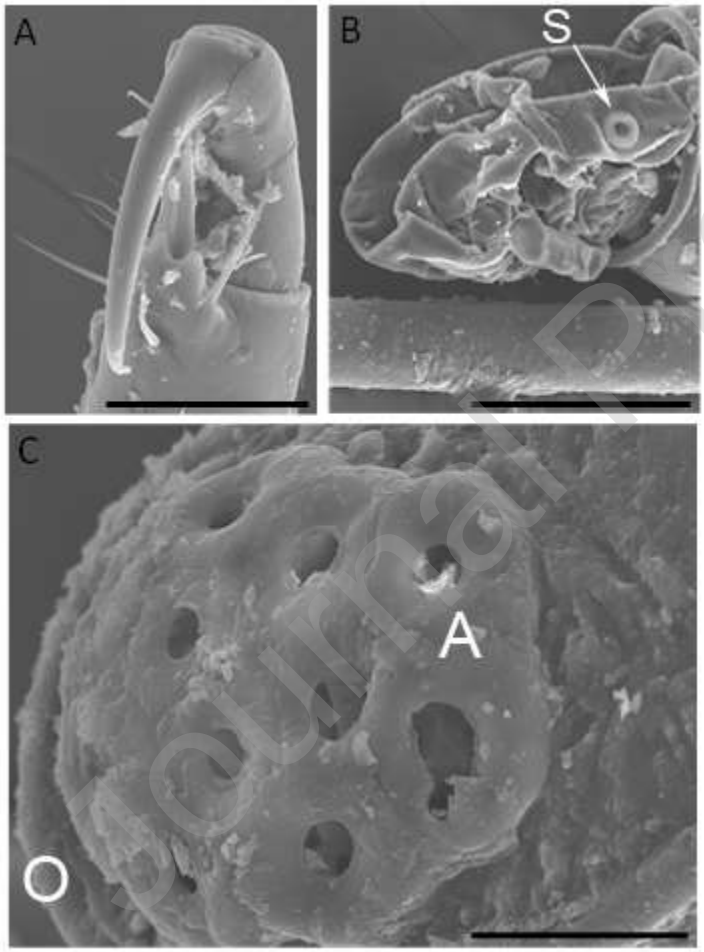

Figure 5. SEM photomicrographs of a specimen of *P. h. capitis.* emerging from an egg. A-Detail of louse claw (scale bar = 100μm); B- The head of an emerging nymph with apparent spiracle (S) (Scale bar = 200μm); C- The operculum (O) of an egg with aeropyles (A) apparent (scale bar = 50μm).

4. DISCUSSION

The use of automontage in visualizing archaeological lice and eggs is effective for specimen identification and sex determination in adult lice and also for identifying the major stages of embryo development within recovered eggs. This technique allows for the simultaneous examination of internal and external morphological features. Automontage is cost-effective for researchers with access to the software and associated hardware as it requires no specialized laboratory equipment or supplies for specimen preparation.

SEM is capable of very high magnifications that facilitate detail definition for anatomical structures (Figure 5). SEM provides the detail necessary for examining morphological features of lice and eggs/nits. Both SEM and automontage are useful for sex estimation in adult lice. SEM is less effective than automontage in the determination of the stages of embryo and nymph development as only external egg morphology is visible in SEM images.

SEM accessibility is often limited, and it may incur significant costs to the researchers for specimen preparation and beam time. Specimen preparation is more involved for SEM than for automontage and may result in specimen loss for other types of analysis due to preparation techniques, such as sputter-coating. However, the use of environmental SEM (ESEM) techniques is much less destructive and may reduce specimen destruction as well as allow for some forms of subsequent analysis (Arriaza et al., 2012, 2013a, 2013b, 2014; Dutra et al., 2014). Using SEM

also requires technical skills that may or may not be possessed by the researchers. In the event that researchers are not SEM trained, there could be additional costs to research projects for employing a SEM technician. The use of other minimally destructive techniques, such as confocal laser scanning microscopy, may also be useful additions to a combined approach when CLSM equipment is readily available and cost-effective for researchers examining these kinds of specimens from archaeological contexts.

To illustrate the information potential of lice, it is instructive to review the research directions of active labs from Table 1. Arriaza et al., (2013b) utilized low and variable pressure modes for SEM. The results showed the structure of louse eggs in dramatic detail. The details of emergent nymphs within fractured eggs were imaged including abdomens, abdominal spiracles, claws, thoraces, and thoracic spiracles (Arriaza et al., 2013b). This group continued to apply a variety of methods to quantify infestations across demographic groups for a population of Chinchorro mummies from Chile (Arriaza et al., 2013a). Stereomicroscopy was used for separating and cleaning hair and to quantify infestations. All separated samples were then observed via light microscopy to determine the sex of adult specimens and to determine whether unhatched eggs contained embryos. A subset of lice was imaged using variable pressure SEM. This research showed that the internal details of the eggs were visible with LM. As a population-based analysis of mummies, this paper is an excellent source for methods of conservation, preparation, and analysis (Arriaza et al., 2013a). These researchers subsequently turned their attention to louse remains on 73 delousing combs found in Chilean tombs (Arriaza et al., 2014). A large sample of lice was found including 14 adults, 33 nymphs and 283 eggs. It is not surprising that some notable specimens were represented in this sample. A remarkable case of a hatching nymph was imaged by SEM. In this image, the operculum with aeropyles is still

associated with the emergent nymph, part of which is still within the egg (Arriaza et al., 2014: Fig. 3). To count the numbers of eggs on the combs, a hand lens was used. Some lice were selected for examination by LM and variable pressure SEM.

Dutra and colleagues (2014) fractured eggs in order to image late stage nymphs. In this study, the authors employed stereomicroscopy followed by compound light microscopy and variable pressure SEM. Importantly, this research team used a simple method of adhesive tape application to remove sections of eggshells, revealing developing embryos within (Dutra et al., 2014). This allowed the researchers to image nearly fully developed embryos within eggs. Following the fracturing of the eggs, the researchers used SEM to image the contents.

Researchers in Israel frequently face the challenges of poor preservation. In 1988, Mumcuoglu and Zias used stereomicroscopy to image a nymph and an egg recovered from delousing combs dated to first century BC to the eighth century AD. Later, Mumcuoglu and Hadas (2011) recovered lice from a Roman Period delousing comb. Using compound light microscopy, they imaged the head of first instar nymph and the apical part of a leg from a first instar nymph including the tarsus, tibia, and femur. Light microscopy was also used to document a louse on a hair discovered in the excavations of Nahal Hemar Cave (Zias and Mumcuoglu, 1991). From the perspective of imaging, a noteworthy paper addresses louse remains found at Masada in association with a group of textiles that is dated to the time of the revolt (Mumcuoglu et al., 2003). In this case, confocal laser scanning microscopy was used to identify key morphological features leading to diagnosis.

Molecular analysis has a role that should be considered when researchers plan analyses of archaeological lice. The application of PCR to well preserved Chiribaya head lice showed that

louse-borne infection routes can be discovered (Aufderheide et al., 2005). In this example, adult lice were tested for the DNA of the protozoa *Trypanosoma cruzi*. Two of 57 lice tested positive for *T. cruzi*. For very badly preserved specimens, combined microscopic and PCR analysis is a demonstrated combination for identifying lice and assessing louse-borne infection (Raoult et al., 2005). Lice from mass burial sediments of Napoleon's soldiers buried in Vilnius, Lithuania were diagnosed by SEM, stereomicrosopy and polymerase chain reaction (PCR) (Raoult et al., 2005). In this case, louse fragments by themselves were insufficient evidence of pediculosis. Stereomicroscopy, SEM and PCR were applied to the same louse samples to verify louse diagnosis. Both lice and teeth from the burials were used to demonstrate louse-borne bacterial infections of *Rickettsia prowazekii* and *Bartonella quintana*. PCR of dental pulp from 35 soldiers revealed DNA of both bacteria. *Bartonella quintana* DNA was recovered from 3 lice. Importantly for this review, stereomicroscopy, SEM and PCR were applied to the same louse samples. This shows the excellent potential of even poorly preserved lice in providing detailed understanding vector borne infections (Raoult et al., 2005).

All of these studies could have been augmented with automontage imaging because automontage is non-destructive path to capture images for archive before proceeding with destructive analysis. Therefore, specimens can be permanently curated after imaging. In combination with imaging, molecular sequencing of pathogen DNA trapped within louse bodies and automontage images of the bodies provides a convincing approach to diagnosis pediculosis and associated vector-borne infections. Because automontage can capture within-egg details of maturing embryos and hatching nymphs, there is less need for laboratory fracturing of eggs to see details within. If applied on a large number of eggs from mummies, future researchers will be able to characterize morphological development of embryos following the stages illustrated

by other researchers (Cueto et al., 2006). Such data could provide insights into the persistence of louse maturation after death of the host.

Importantly, the automontage images will translate well to magazine coverage of research for the general public. The ability to capture images of action, such as egg hatching, are illustrative of life at the microscopic scale that capture the imagination of science-oriented people.

We believe that we have only begun to explore the application of automontage to archaeological louse studies. As instrumentation becomes more widely available, we anticipate a broader use of the methods will result in further exploration of epidemiology of louse infestation, potential transmission of microparasites, and evolution of louse morphology. In conclusion, we emphasize that future studies of ancient lice employ a combination approach as recommended by Arriaza (2011). We find that combined automontage and SEM techniques employed sequentially maximize the data that can be collected from these types of specimens.

Declaration of interests

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.

5. ACKNOWLEDGEMENTS

The mummy recovery was accomplished with NSF funding to Dr. Jane Buikstra (BNS89-20769). The authors would like to thank the University of Nebraska-Lincoln Undergraduate Creative Activities and Research Experiences Program (UCARE) for lab support. The SEM

imaging was done in the University of Nebraska-Lincoln, Center for Biotechnology, Morrison Microscopy Core Research Facility. We thank Christian Elowsky and Han Chen for their assistance in image procurement and figure development. We also thank the School of Natural Resources within the University of Nebraska-Lincoln. Automontage images in this research were taken in the Biodiversity Synthesis Laboratory of the University of Nebraska State Museum (NSF/BSandI Multi-user Equipment grant DBI 0500767 to M. L. Jameson and F. C. Ocampo).

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