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EXPERIMENTAL MATURATION OF FEATHERS: IMPLICATIONS FOR INTERPRETATIONS OF FOSSIL FEATHERS

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ABSTRACT: **Exceptionally preserved fossil feathers and feather-like integumentary structures provide valuable insights into the early evolution of feathers and flight, but taphonomic biases often make interpretations at the microstructural and ultrastructural levels ambiguous. Maturation experiments have been demonstrated to be useful for investigating the taphonomic alterations of soft tissues, including feathers, during diagenesis. However, experimentally matured feathers resembling fossil feathers preserving keratinous matrix have not yet been obtained. Here we employ experimental maturation to obtain feathers corresponding to different degradation stages, and compare these matured feathers with untreated feathers and fossil feathers at the macroscopic, microstructural, and ultrastructural levels. Results show that several features of thermally matured feathers are similar to those found in fossil feathers. The fusion of barbules that occurred in thermally matured feathers suggests that such a process could occur during diagenesis, making barbules difficult to identify in fossil feathers. Under the most extreme experimental condition, the keratinous matrix can partially survive when the whole feather is turned into ash-like remains and many melanosomes are exposed. Moreover, our results show that the keratinous matrix immediately surrounding melanosomes appears to be more resistant to degradation than the unpigmented keratinous matrix, supporting the hypothesis that melanin can act as a fixative agent to prevent the degradation of keratin.**

INTRODUCTION

Feathers are complex integumentary derivatives with hierarchical branches of rachis, barbs, barbules, and barbicels. The discoveries of exceptionally preserved feathers and feather-like integumentary structures in non-avian dinosaurs and early birds have provided valuable information on the early evolution of feathers and flight (e.g., Xu 2006; Perrichot et al. 2008; Xu and Guo 2009; Xu et al. 2014; Feo et al. 2015; McNamara et al. 2018). However, feathers must have been altered by taphonomic processes, similarly to other soft tissues, before being discovered as fossils (Benton et al. 2008; McNamara et al. 2013; Sansom 2014; Colleary et al. 2015). One consequence of taphonomic alterations is that interpretations of fossil feather structures at microstructural and ultrastructural levels are often ambiguous. For example, the interpretations of melanosomes and keratinous tissues in fossil feathers continue to be debated (e.g., Moyer et al. 2014; Lindgren et al. 2015a; Schweitzer et al. 2015; Vinther 2015; Moyer et al. 2016; Pan et al. 2016; Vinther 2016; Saitta et al. 2017a, 2017b, 2018; Schweitzer et al. 2018; Pan et al. 2019).

Nevertheless, experimental studies of taphonomic alterations can help to better interpret fossils (e.g., Sansom 2014; Briggs and McMahon 2016; Purnell et al. 2018). Using maturation experiments, McNamara et al. (2013) demonstrated that melanosome size can be altered by elevated temperature and pressure during diagenesis, which has implications for the reconstructions of the coloration of fossil feathers based on the shape and size of fossil melanosomes (e.g., Li et al. 2010, 2012; Carney et al. 2012). Saitta et al. (2019) showed that the maturation of feathers under high pressure and temperature can expose densely packed melanosomes, as in fossil feathers (Colleary et al. 2015), but whether keratinous tissues survived or not was not specifically examined. Moyer et al. (2016) and Schweitzer et al. (2018) showed that the maturation of feathers under high temperature can lead to the degradation of melanosomes when keratin epitopes can still be detected with immunohistochemistry, comparable to the condition of fossil feathers from Holocene high-temperature hot spring deposits (Channing et al. 2005; Schweitzer et al. 2018). Recent studies showed that aside from melanosomes, keratinous tissues can also be preserved in Mesozoic fossil feathers and feather-like structures (Schweitzer et al. 1999; Pan et al. 2016, 2019; Yang et al. 2019), but experimentally matured feathers resembling such fossils have not yet been obtained.

Here we employ experimental maturation to investigate how feather details change at the macroscopic, microstructural, and ultrastructural levels. We compare the results with the feathers of *Eoconfuciusornis*, an Early Cretaceous bird, from which evidence of keratin has been reported (Pan et al. 2016).

MATERIAL AND METHODS

The principle of experimental maturation in simulating diagenesis is that elevated temperatures can accelerate the chemical reactions that may take millions of years during diagenesis (e.g., Briggs and McMahon 2016; Saitta et al. 2019). Because geologic processes are complex, maturation experiments are not a replication of natural diagenesis (Gupta et al. 2007). It is therefore important to examine whether maturation experiments can generate features resembling those found in fossils. In this study, the results obtained were compared with fossil feathers of *Eoconfuciusornis* (STM7-144) (Zheng et al. 2017). The placement of foil-wrapped feathers in water

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pressurized autoclaves is geologically realistic in that the sediment in which feathers are buried in natural systems may be saturated with water.

Different taphonomic processes may lead to different preservation states among fossil feathers. Thus, we adjusted the experimental settings to obtain feathers exhibiting different degradation stages. Four flight feathers in total from a domestic chicken (*Gallus gallus*) were wrapped in aluminum foil, placed in four separate water-pressurized autoclaves, and matured in a single furnace under four different conditions: (1) 250 bars, 100°C for one hour; (2) 250 bars, temperature gradually increased from 100°C to 150°C over one hour and kept at 150°C for one hour; (3) 250 bars, temperature increased from 150°C to 200°C over one hour and kept at 200°C for one hour; and (4) 250 bars, temperature increased from 200°C to 250°C over one hour and kept at 250°C for one hour. To clarify, the feather under condition 1 was matured for one hour while the others were matured for two hours. These experimentally matured feathers were unwrapped and photographed before being air-dried in a fume hood. An untreated flight feather from the same chicken was used as a control.

Small pieces cut from the untreated and treated feathers were mounted on stubs with carbon tape and coated with gold before being observed and photographed using a Field Emission Scanning Electron Microscope (LEO 1530VP). For transmission electron microscopy observations, smaller pieces cut from untreated and treated feathers were embedded in LR White; 70-nm-thick sections were made using a Leica EM UC7 ultramicrotome with a DiATOME 45° knife. These sections were mounted on carbon-coated copper grids and observed using a Transmission Electron Microscope (JEM-1400).

RESULTS

Preliminary Observations

Photos were taken immediately after unwrapping the aluminum foil, and thus the features of each of the treated specimens were documented before the specimens were air-dried (Fig. 1). There appears to be no obvious difference between the feather treated under condition 1 and the control feather. The feathers treated under condition 2 and condition 3 are plastic, as indicated by the folds. There is a yellowish liquid around the feather treated under condition 3, which is not found under either condition 1 or condition 2. The feather treated under condition 4 only left some ashes and a small amount of yellowish liquid.

Scanning Electron Microscopy (SEM) Observations

SEM observations show that the experimental treatments gradually make the overall feather morphology less distinct at a microstructural level (Fig. 2). No obvious alteration was observed on the feather treated under condition 1 (Fig. 2B), compared with the untreated feather (Fig. 2A). Under condition 2, the barbules and barbicels remain visible (Fig. 2C, 2D). Fusion of the barbules occurred under condition 3 (Fig. 2E, 2F) and the barbicels became hard to identify (Fig. 2F). Under condition 4, only some fragments of the feather were left, with the original structures becoming difficult to identify (Fig. 2G, 2H).

Cracks were documented in the feathers treated under condition 2 (Fig. 2C) and condition 3 (Fig. 2E), but not condition 1. The feather treated under condition 3 is more densely cracked than that under condition 2. In both samples, the cracks of the barbs are largely perpendicular to the long axes of the barbs. The cracks of the barbules occur at some distance from the roots of the barbules, leaving parts of the barbules attached to the barbs.

At higher magnifications, more details of the structural degradation were documented (Fig. 3). The surfaces of the barbules in the untreated feather have protuberances and grooves (Fig. 3A). These protuberances and grooves remain intact under condition 1 (Fig. 3B). Under condition 2, the surfaces of barbules became wrinkled and pitted, with melanosomes occasionally exposed (Fig. 3C). Under condition 3, the original protuberances and grooves vanished entirely; melanosomes are more frequently exposed (Fig. 3D). Under condition 4, significant amounts of melanosomes are fully exposed, while some are embedded in the altered keratinous matrix (Fig. 3E, 3F).

The feathers treated under condition 3 and condition 4 exhibit features comparable to those of the feathers of *Eoconfuciusornis* (Fig. 4). Figure 4A shows a feather of *Eoconfuciusornis* that exhibits a similar pattern of cracking to that observed in the feather treated under condition 3 (Fig. 4B). In this fossil feather, the margins of barbules are indiscernible, as are the fused barbules in the feather treated under condition 3. Figure 4C shows a plate-like feather fragment of *Eoconfuciusornis*, which is too large to be from one barbule (see Pan et al. 2016, fig. 1 for location of this sample). It is likely a result of fusion, but in a greater degree than that under condition 3 (Fig. 4D). Figure 4E shows structures from a feather of *Eoconfuciusornis* that are similar to the granular keratinous remnants in the feather treated under condition 4 (Fig. 4F).

Transmission Electron Microscopy (TEM) Observations

TEM observations (Figs. 5, 6) provide additional details from a cross-section perspective complementing SEM observations. Under TEM, almost no differences were observed between the untreated feather (Fig. 5A) and the feather treated under condition 1, except for transverse stripes that formed at the barbules under condition 1, indicating slight localized shrinkage of the keratinous matrix (Fig. 5B, at arrow in 5B inset). The feather treated under condition 2 still retains the contours of the barbules, but exhibits many notches on the margins of the barbules, some of which reach the melanosomes (Fig. 5C). Under condition 3, the original contours of the barbules were completely lost, with many melanosomes partly embedded in the keratinous matrix (Fig. 5D). The keratinous matrix immediately surrounding the melanosomes appears more resistant to degradation, as indicated by the phenomenon that the keratinous tissues formed a membrane-like structure (arrows in Fig. 5D inset) surrounding the melanosomes when the keratinous matrix farther from the melanosomes were degraded to a deeper depth to form notches (arrowheads in Fig. 5D inset). The remains of the feather treated under condition 4 were not observed via TEM, as many melanosomes are fully exposed.

Besides the microstructural alterations to the keratinous matrix, TEM observations reveal the degradation process experienced by melanosomes within the keratinous matrix (Fig. 6). The melanosomes in chicken feathers may have one to several small internal voids (Fig. 6A). Similarly, internal voids were observed in the melanosomes in *Eoconfuciusornis* feathers (Pan et al. 2016, fig. 6a). The degradation of melanosomes in our experiment compares well with the *in vivo* degradation of melanosomes in the retinal pigmented epithelium of the opossum (Herman and Steinberg 1982, figs. 15–17). As described by Herman and Steinberg (1982), three stages of degradation can be identified in the feathers treated under condition 2 and condition 3: (1) melanosomes are frayed at the edges, some of them with melanin granules scattered around the periphery (Fig. 6C); (2) melanosomes become less electron-dense, as indicated by the lighter color relative to the dark part in melanosomes with an uneven color (Fig. 6D); and (3) melanosome residues blend with the keratinous matrix, as indicated by the indistinct margin (Fig. 6D). In the feather treated under condition 2, melanosomes at stage 1 are relatively common (5 out of 17 in Fig. 6C). In the feather treated under condition 3 (Fig. 6D), most of the observed melanosomes are at stage 2, with very few at stage 3. The concentration of melanosomes appears to increase with maturation temperature (1.17/μm^2 in the untreated feather, 2/μm^2 in the feather treated under condition 1, 2.38/μm^2 in the feather treated under condition 2, and 3.4/μm^2 in the feather treated under condition 3) (Online Supplemental File). Especially, in the feather treated under condition 4, many exposed melanosomes are no longer separated from each other by the keratinous matrix (Fig. 3F).

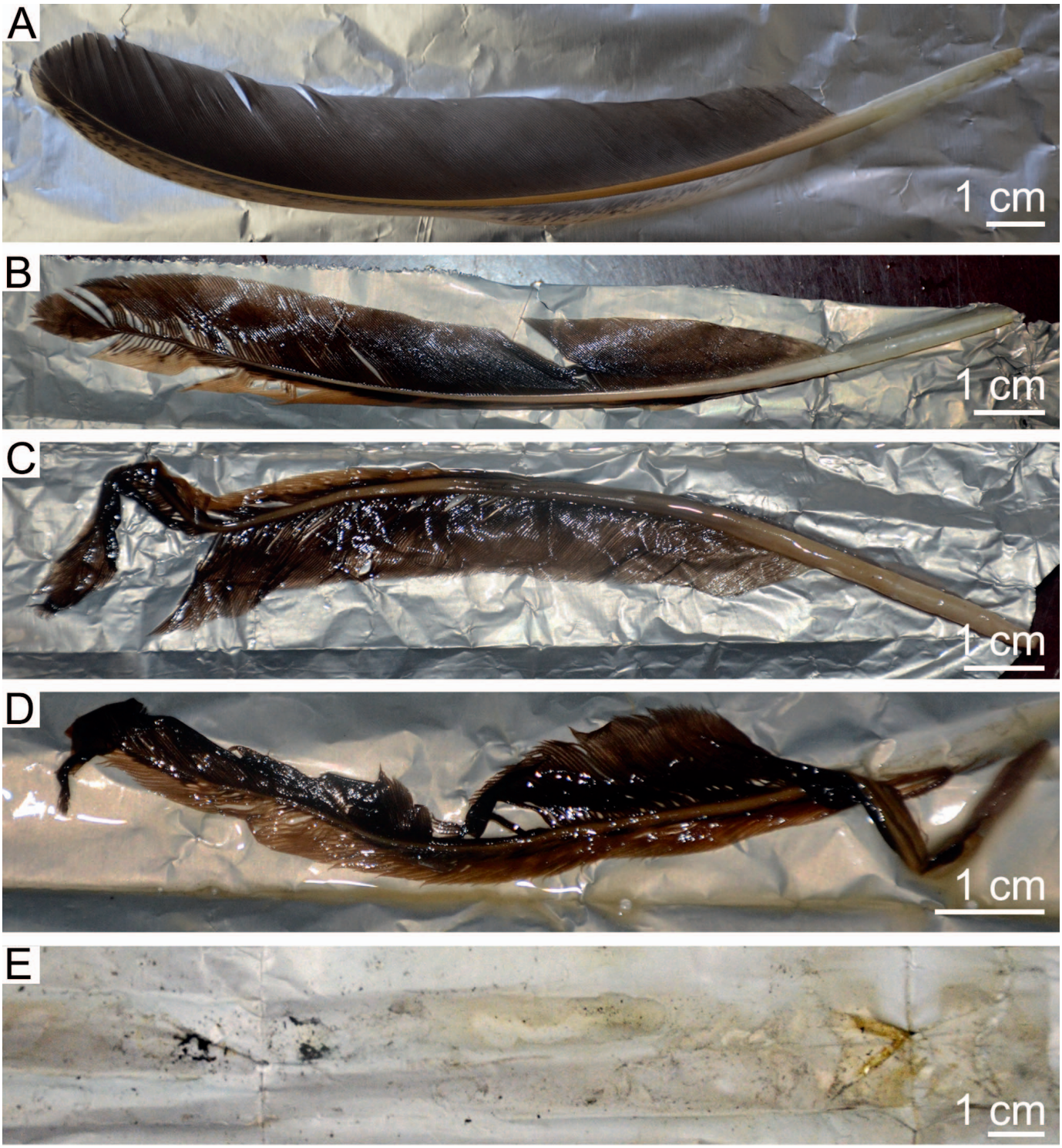
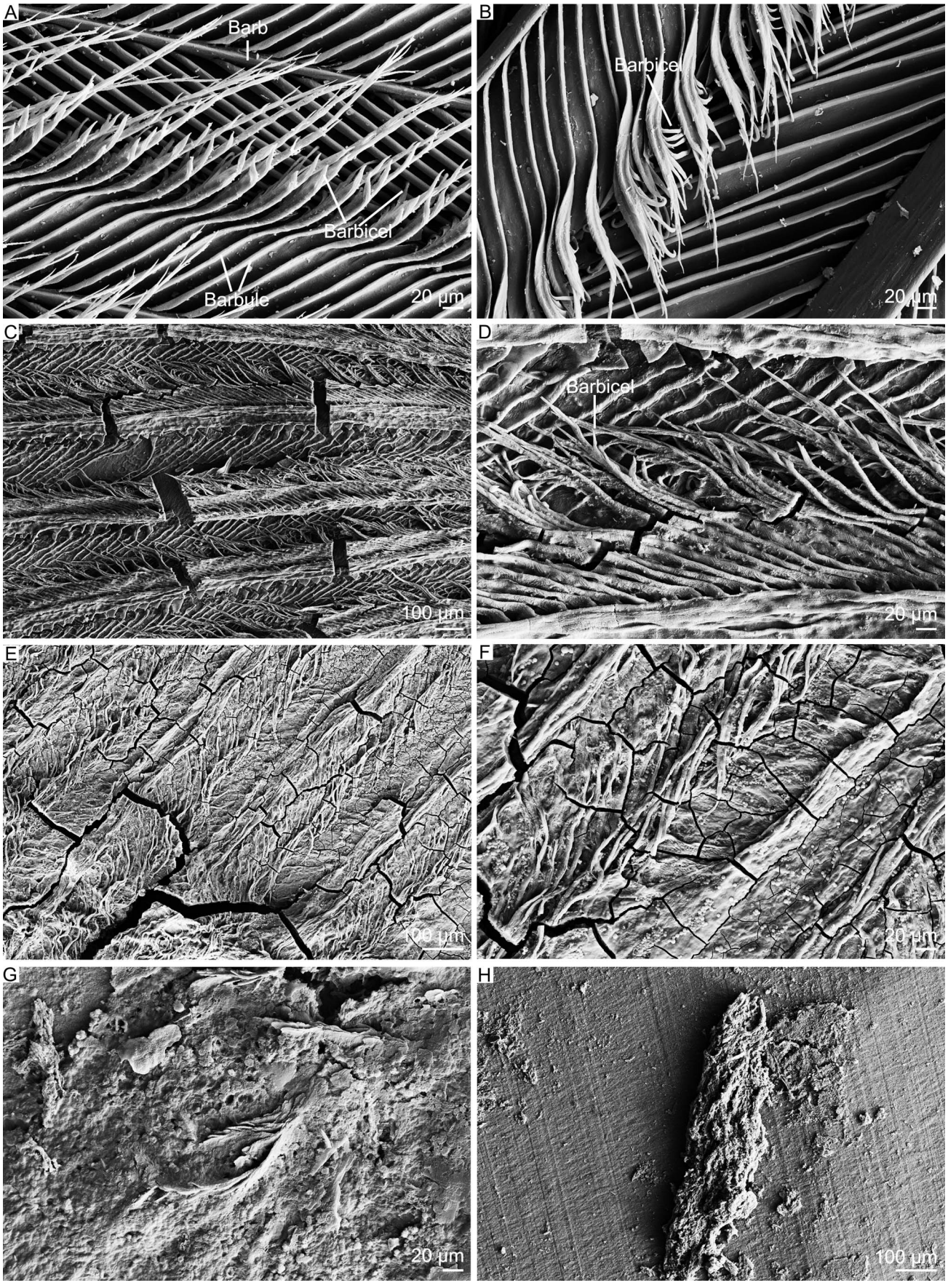


FIG. 1.—Photos of untreated and treated feathers. **A**) Untreated feather. **B**) Feather treated under condition 1. **C**) Feather treated condition 2. **D**) Feather treated under condition 3. **E**) Feather treated under condition 4.

DISCUSSION

Fusion of Barbules

The appearance of barbules is one of the major events in feather evolution (Prum 1999; Xu 2006; Xu and Guo 2009). The interlocking of barbules forms the closed pennaceous vanes in contour feathers, including flight feathers. Although it has long been recognized that barbules are hard to identify in fossils (Zhang et al. 2006; Xu and Guo 2009; Gren et al. 2017), the mechanisms underlying why this is the case remain unclear. Our observations on treated feathers and fossil feathers (Fig. 4A, 4C) suggest that the difficulty in identifying barbules in fossils can be caused by the



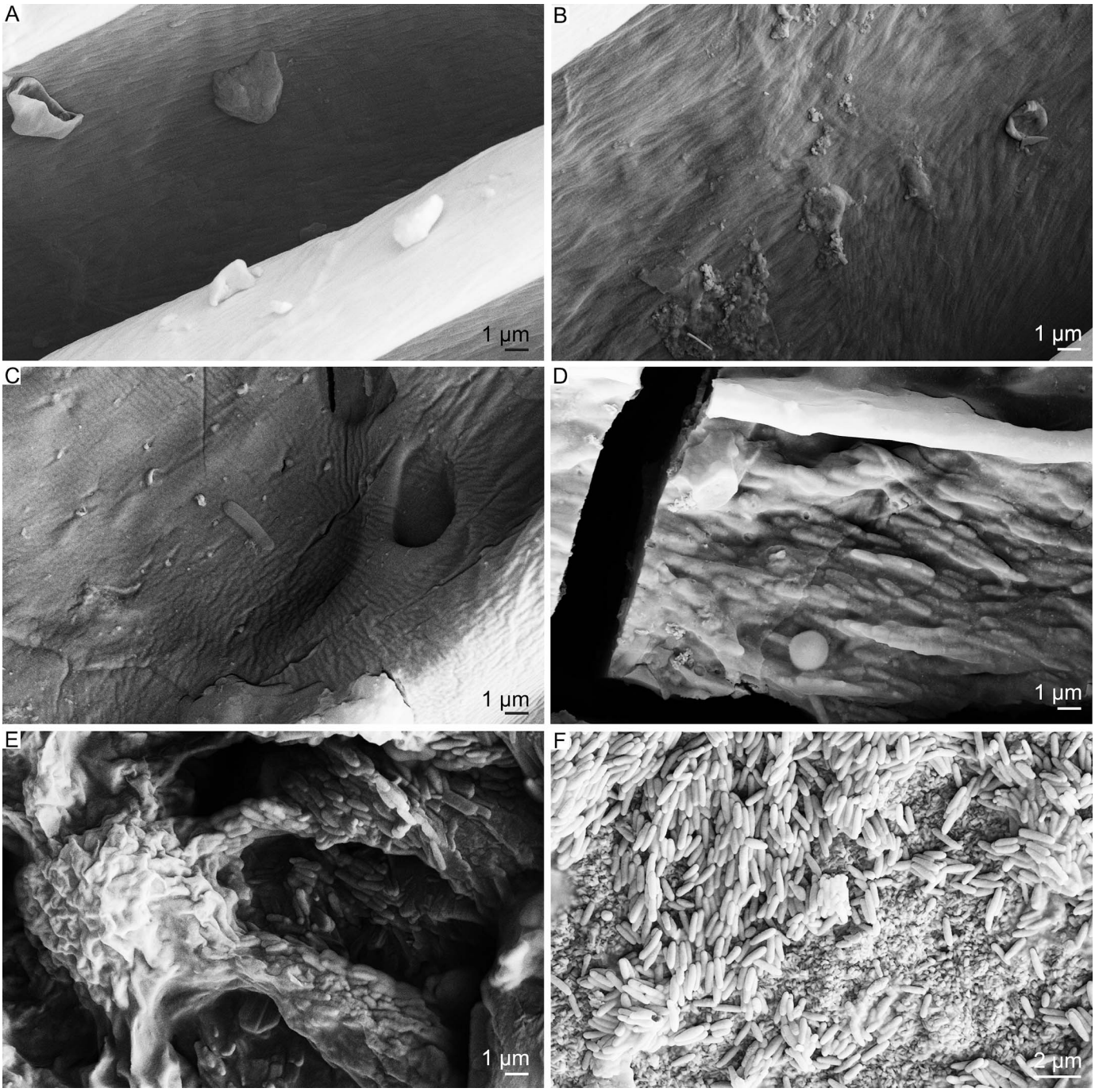


FIG. 3.—SEM images of untreated and treated feathers showing the inward degradation of the barbules. **A**) Untreated feather. **B**) Feather treated under condition 1. **C**) Feather treated under condition 2. **D**) Feather treated under condition 3. **E**, **F**) Feather treated under condition 4.

fusion of barbules. In fact, Saitta et al. (2017b) observed that barbs and barbules had begun to fuse together during decay of feathers and maturation greatly intensified this fusion (Saitta et al. 2017b, figs. s11, s22). However, they thought that such information was unlikely to be recorded in fossils, because they had not observed fused barbules in fossils (Saitta et al. 2017b). The fused barbules in the feathers of *Eoconfuciusornis* (Fig. 4) highlight the potential of exceptionally preserved fossils to increase our knowledge. Therefore, we suggest that the fusion of barbules and potentially other keratinous structures merits consideration when interpreting the structures of fossil feathers.

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FIG. 2.—SEM images of untreated and treated feathers showing the overall changes of feathers. **A**) Untreated feather. **B**) Feather treated under condition 1. **C**, **D**) Feathers treated under condition 2. **E**, **F**) Feather treated under condition 3. **G**, **H**) Feather treated under condition 4.

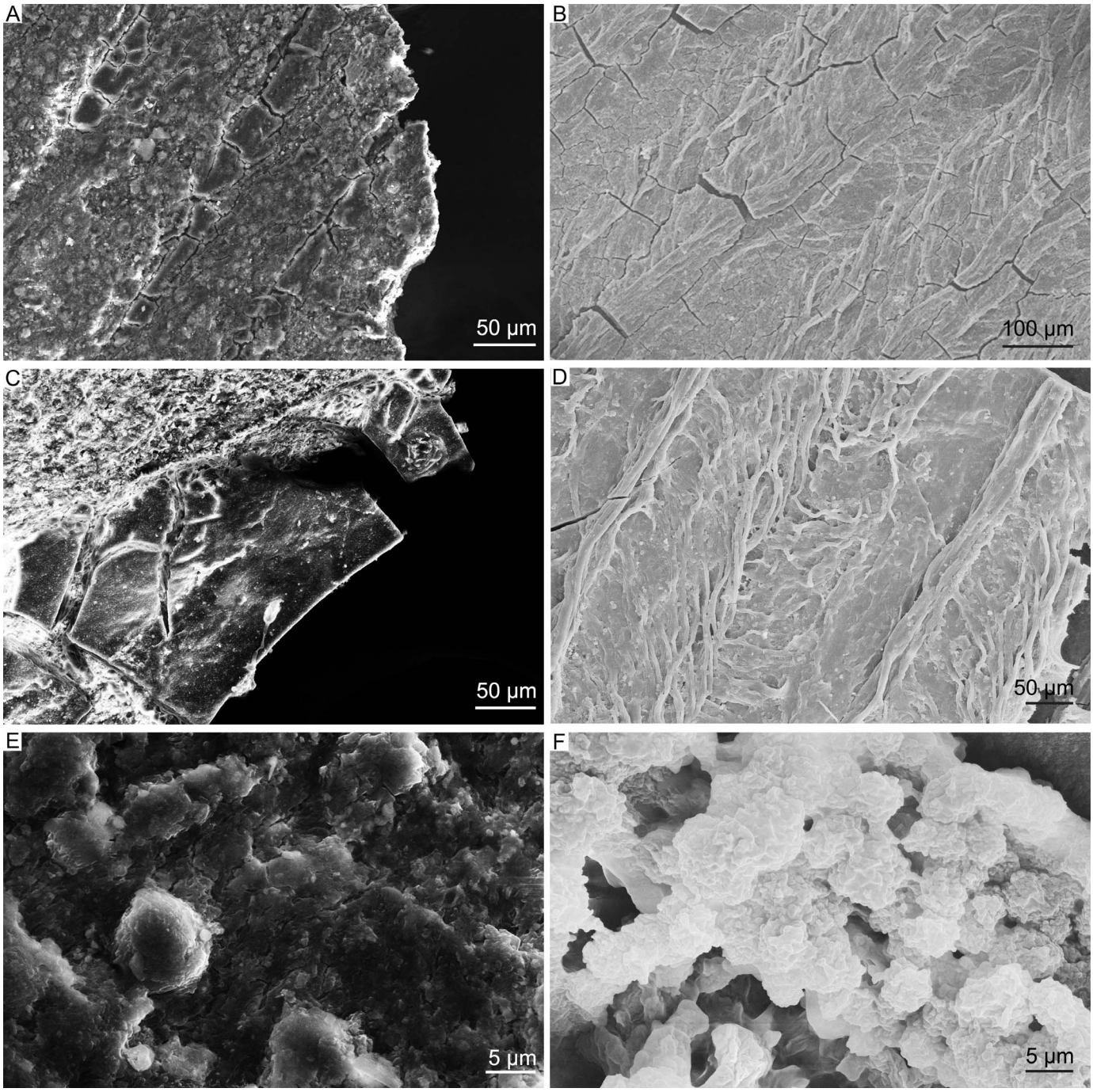

FIG. 4.—Comparison of the feathers of *Eoconfuciusornis* and treated feathers. **A**, **C**, **E**) Feathers of *Eoconfuciusornis*. **B**, **D**) Feather treated under condition 3. **F**) Feather treated under condition 4.

Spatial Packing of Melanosomes

The microbodies in fossil feathers are often densely packed (e.g., Vinther et al. 2008, 2010; Zhang et al. 2010; Li et al. 2012; Vitek et al. 2013; Hu et al. 2018). By contrast, the melanosomes in extant feathers are often sparse and non-overlapping (Moyer et al. 2014), except in iridescent feathers (Hu et al. 2018). Consequently, these microbodies in fossil feathers were argued to be more consistent with microbes (Moyer et al. 2014), as microbes can be densely packed and overlap in their size and shape with melanosomes (Knight et al. 2011; Moyer et al. 2014; Lindgren et al. 2015a; Schweitzer et al. 2015). To date, chemical evidence has been used to support the interpretation of the microbodies in fossil feathers as melanosomes (Colleary et al. 2015; Lindgren et al. 2015b; Pan et al. 2016, 2019; Gren et al. 2017). The dense packing of the melanosomes in fossil feathers was hypothesized to be due to diagenetic alterations. Both previous experiments (Saitta et al. 2019) and our experiments show that the dense packing of melanosomes (Fig. 3F) can originate via degradation of the keratinous matrix enveloping the melanosomes by thermal maturation under high pressure and temperature, supporting the hypothesis that

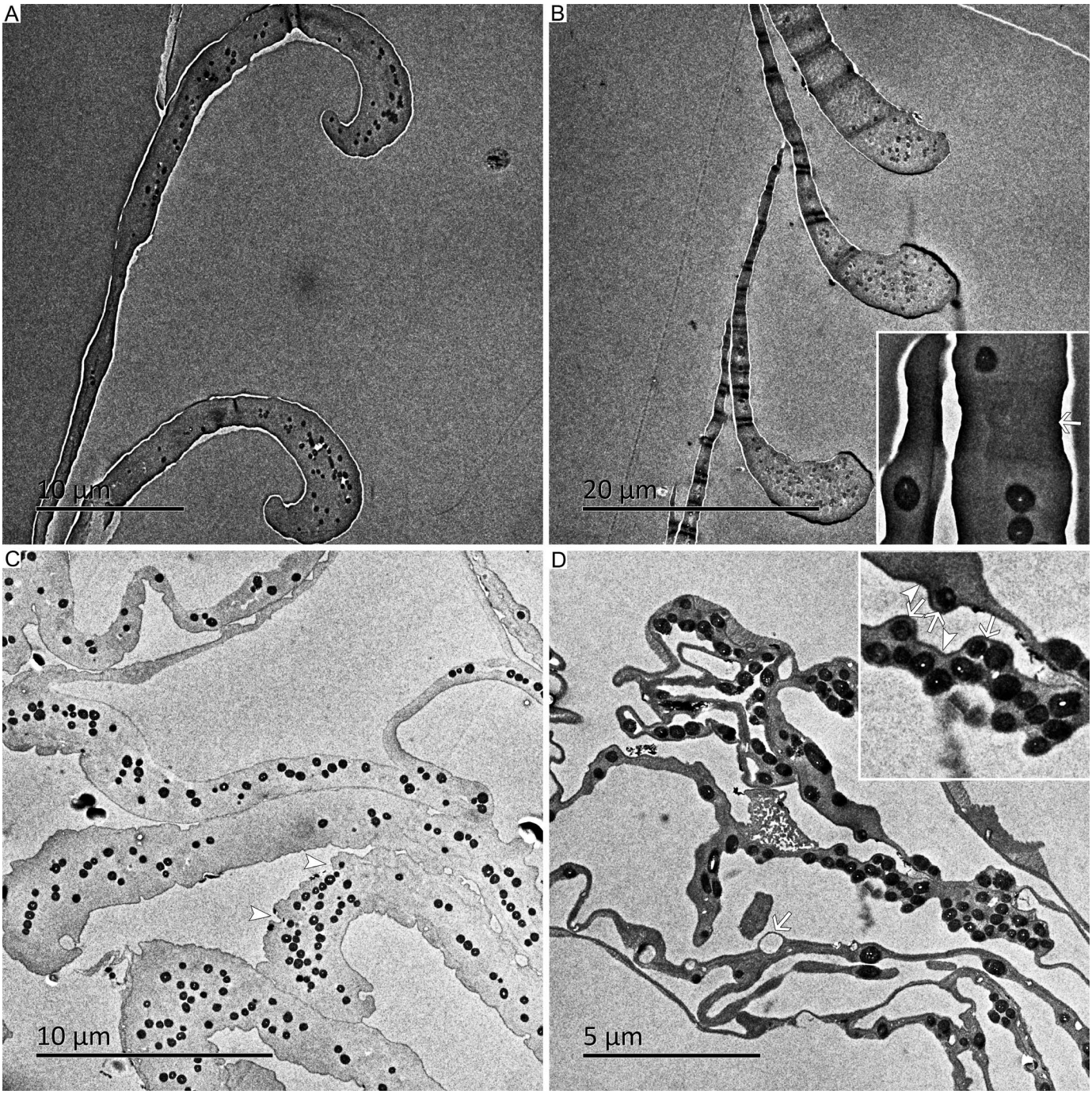

FIG. 5.—TEM images of untreated and treated feathers showing the inward degradation of the barbules. **A**) Untreated feather. **B**) Feather treated under condition 1. Inset shows the shrinkage of the keratinous matrix (arrow). **C**) Feather treated under condition 2. **D**) Feather treated under condition 3. Inset shows that the keratinous tissues formed a membrane-like structure (arrows) surrounding the melanosomes when the keratinous matrix farther from the melanosomes were degraded to a deeper depth to form notches (arrowheads).

diagenesis can lead to a greater concentration and closer packing of melanosomes.

Aligned melanosomes have been documented in many fossil feathers (e.g., Vinther et al. 2008, 2010; Zhang et al. 2010; Li et al. 2012; Vitek et al. 2013; Hu et al. 2018). Similarly, the fully exposed melanosomes in our experiments also show some degree of alignment (Fig. 3F), which is likely because the high pressure (250 bars) during experiments made the aluminum foil packed tightly enough to hold the melanosomes largely *in situ*. The tight packing of the aluminum foil during experiments is also illustrated by the aforementioned yellowish liquids held in association with the matured samples treated under condition 3 and condition 4 (Fig. 1).

Preservation of Keratinous Tissues and Melanosomes in Fossils

In terms of the preservation potential of keratinous tissues and melanosomes in fossils, two opposing views were proposed based on

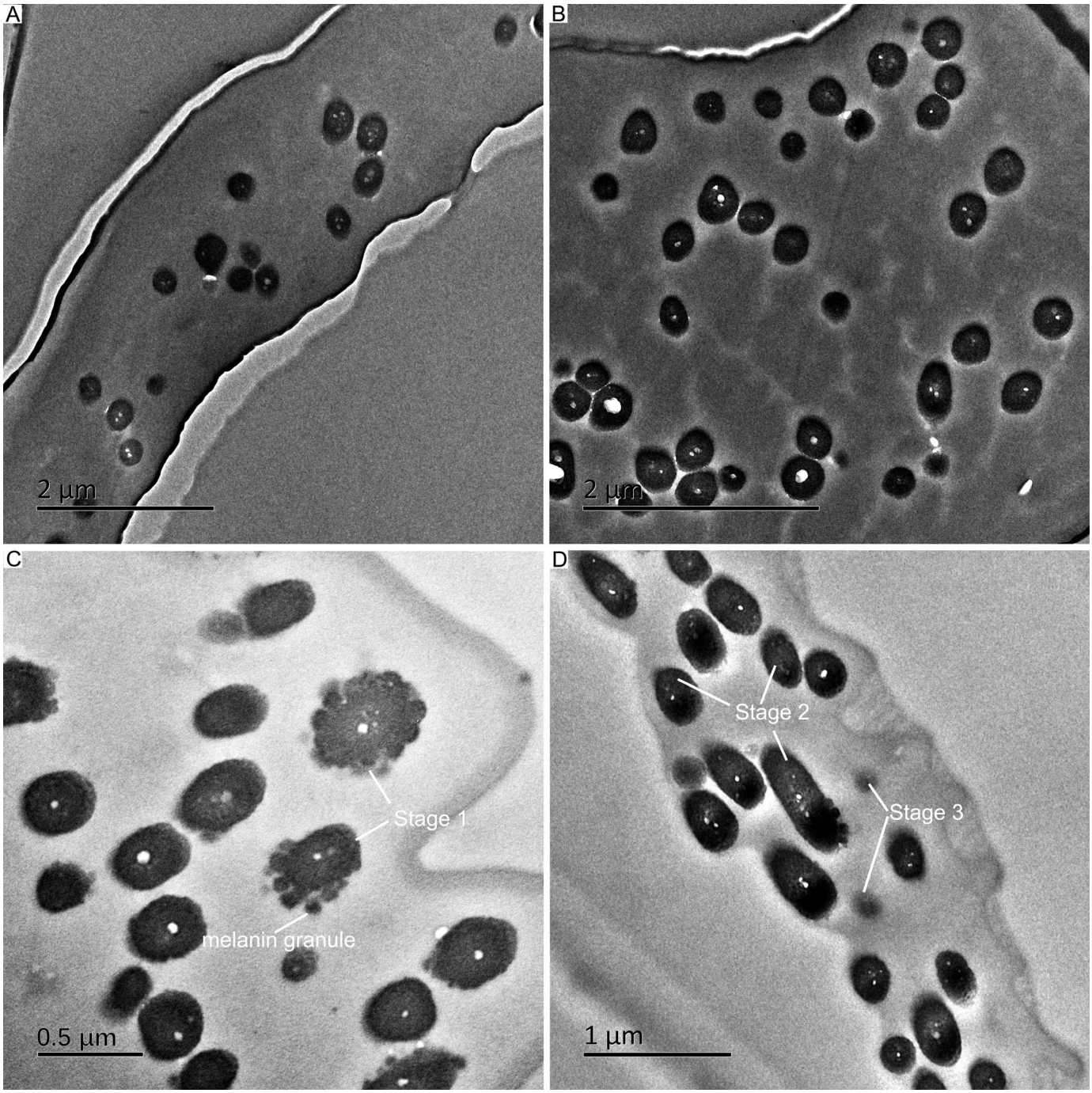

FIG. 6.—TEM images of untreated and treated feathers showing the degradation of the melanosomes. **A**) Untreated feather. **B**) Feather treated under condition 1. **C**) Feather treated under condition 2. **D**) Feather treated under condition 3.

maturation experiments (Moyer et al. 2016; Saitta et al. 2017a, 2017b, 2019; Schweitzer et al. 2018). Moyer et al. (2016) and Schweitzer et al. (2018) showed that after 10 years of maturation, keratin epitopes can survive but melanosomes were not observed. Accordingly, it has been argued that keratinous tissues are durable and have higher preservation potential than melanosomes, which is supported by analyses of fossil feathers from Holocene high-temperature hot spring deposits (Moyer et al. 2016; Schweitzer et al. 2018). Conversely, Saitta and colleagues (Saitta et al. 2017a, 2017b, 2019) held the view that keratinous tissues are unlikely to be preserved in fossils and the only remaining components of fossil feathers could be calcium phosphate (original or of diagenetic origin) and pigments; original calcium phosphate has been found in the feather calamus (Blakey et al. 1963; Pautard 1963). They based their arguments mainly on their failure to find keratin markers in two Eocene fossils with Py-GC-MS and their success in degrading keratin but retaining melanosomes via thermal maturation.

The differences in experimental settings among these experiments suggest that the relative preservation potential of keratinous tissues versus

melanosomes is condition-dependent. One major difference is that pressure was not elevated during the maturation experiments of Moyer et al. (2016). This is actually not unrealistic (*contra* Saitta et al. 2019), because such settings can occur in natural environments, as indicated by the fossil feathers from the Holocene sinters (Schweitzer et al. 2018). However, feathers preserved in the sinter deposits are not common in the fossil record, and the results of Moyer et al. (2016) cannot explain the close packing of melanosomes in other fossil feathers (e.g., Vinther et al. 2008, 2010; Colleary et al. 2015; Pan et al. 2016, 2019). As shown by the experiments of Saitta et al. (2019) and our experiments, the close packing of melanosomes can originate via degradation of the keratinous matrix enveloping the melanosomes (Fig. 3F) by thermal maturation of feathers under high pressure and temperature. Because keratinous tissues are less durable than melanosomes under high pressure and temperature, the preservation of keratinous tissues in fossil feathers requires less harsh environments than preservation of melanosomes. However, the failure to find keratin signals in some fossils (Saitta et al. 2017a) cannot preclude the identification of keratin signals in other fossils (Pan et al. 2016, 2019); this discrepancy can be related to the differences in taphonomic histories and the method used by Saitta et al. (2017a)—keratins and melanins yield similar pyrolysis compounds when analyzed using Py-GC-MS.

It has been suggested that melanin can act as a fixative agent to prevent degradation of keratin (Moyer et al. 2016). Consistent with this hypothesis, Saitta et al. (2017b) found that melanized feathers were degraded to a lesser extent than non-melanized feathers after maturation. Moyer et al. (2016), however, observed no differences in structural integrity between melanized and non-melanized regions of the feathers in their experiment. Our TEM observations show that the keratinous matrix immediately surrounding the melanosomes appears to be more resistant to degradation than other parts (Fig. 5D). Moreover, we show that the melanin granules can leach out of the melanosomes to disperse into the keratin matrix (Fig. 6C), which can facilitate chemical reaction between keratin and melanin. Chemical analyses are required to obtain more insights into the mechanism.

CONCLUSIONS

We show that maturation experiments can produce partly degraded feathers that strongly resemble fossil feathers in which keratinous tissues are preserved. These similarities illustrate the relevance of our results to the interpretation of fossil feathers. Based on the comparison of fossil feathers and treated feathers, we suggest that the fusion of barbules can make barbules difficult to identify in fossil feathers and create taphonomic artifacts. Maturation of feathers under high pressure and temperature can produce densely packed melanosomes, comparable to fossil feathers, which suggests that melanosomes are generally more durable than keratinous tissues during diagenesis, and that the preservation of keratin in fossil feathers requires less harsh environments than preservation of melanosomes. Nevertheless, the keratinous matrix can partially survive when the whole feather is turned into ashes and many melanosomes are exposed. Moreover, our results show that the keratinous matrix immediately surrounding melanosomes appears to be more resistant to degradation, supporting the hypothesis that melanin can act as a fixative agent to prevent the degradation of keratin. Future chemical analyses of experimentally matured feathers will provide more information for comparative studies with fossil feathers.

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SUPPLEMENTAL MATERIAL

Data are available from the PALAIOS Data Archive: https://www.sepm.org/supplemental-materials.

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