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Early detritivory and sedimentivory in insects based on *in situ* gut contents from Triassic aquatic nymphs

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Abstract: The colonization of freshwater by insects is one of the milestones in the establishment of continental ecosystems and, thus, of life on our planet. However, several key aspects of this process such as patterns of origination, early adaptations and palaeoecological relationships of the groups involved remain poorly known, namely due to the scarcity of significant assemblages. The Palaeozoic fossil record of freshwater insects is poor and unstudied in suitable detail. Here we analyse exceptionally preserved, three-dimensional cololites (*in situ* gut contents) in abundant mayfly nymphs from Pedra Alta (lower Anisian, lowermost Middle Triassic; Mallorca, Spain), which probably inhabited lentic waters (pools) in a riverine ecosystem. This Konservat-Lagerstätte shows an aquatic insect assemblage *c.* 2 myr older than the similar locality of Grès à Voltzia (Northern Vosges, France). Detailed morphological

and elemental analysis show that the cololites are composed of the same very fine-grained claystone as the fossil-bearing rock. This study presents the oldest direct evidence of insect detritivory, as well as most probably that of sedimentivory. The trophic niche represented by insect sedimentivory in the early continental aquatic ecosystem of Pedra Alta is not known for the subsequent *c.* 240 myr of insect evolutionary history and up to the present. This lifestyle in extant insects is extremely infrequent and is known only in a few species of burrowing mayfly nymphs. Our findings illuminate the role of insects in detritus processing in relatively complex food webs shortly after the end-Permian mass extinction event.

Key words: insect evolution, palaeoecology, trophic net, detritivory, sedimentivory, Mesozoic.

ONE of the milestones in the history of continental ecosystems is the colonization of the continental waters by insects. The Palaeozoic record of aquatic insects is scarce and often ambiguous, with the first records of aquatic insects not known until the late Permian (Wootton 1988; Sinitshenkova 2002, 2003; Zherikhin 2002). Insects play a crucial role in extant aquatic ecosystems, including in key aspects of trophic nets, as recyclers of organic matter (Cummins 1973). The available fossil record indicates that such ecological predominance had already been achieved by, at least, the Middle Triassic (Anisian) based on the rich insect assemblage from the Grès à Voltzia locality in France (Sinitshenkova *et al.* 2005; Lukashevich *et al.* 2010). This view was recently supported by a preliminary palaeoecological assessment of the aquatic insect fauna from the slightly older Pedra Alta

deposit in Spain (Matamales-Andreu *et al.* 2021). A few other localities of similar age are known, but these are either very poor in aquatic insects (e.g. Sinitshenkova *et al.* 2015) or lack them, at least for certain (e.g. Bashkuev *et al.* 2012). During the Triassic, continental ecosystems underwent a profound remodelling largely due to biotic turnovers, and many of the key structural features of modern ecosystems became established (Sinitshenkova 2002; Benton & Wu 2022), especially for the aquatic ones (Zhao *et al.* 2020). Changes in the insect palaeofaunas around the end-Permian mass extinction and the subsequent early recovery are poorly known due to the lack of Lower Triassic palaeoentomological outcrops (but see Briggs & Gall 1990). This gap has been considered one of the most important in the fossil insect record (Zherikhin 2002).

Exceptional preservation deposits provide high-quality ecological data on past ecosystems, mostly those related to aquatic (i.e. compression deposits: e.g. Gall 1972; Kopylov *et al.* 2020) or forested environments (i.e. amber: e.g. Grimaldi 2000; Sánchez-García *et al.* 2021). Even though the evolution of larval stages in insects has classically been one of the most neglected topics in (palaeo)entomology (Peñalver & Pérez-de la Fuente 2014), compression deposits provide rich assemblages of aquatic preimaginal stages of insects, including egg lays, larvae, nymphs, or exuviae. Because compression deposits (particularly those offering exceptional preservation) have their origin in aquatic environments, they are crucial to understand some of the key topics in the evolution of insects in freshwater habitats (Martínez-Delclòs *et al.* 2004). In the case of Triassic insects, virtually known only as compression fossils, the studies are scarce and our knowledge on them is not based on strong taphonomic analyses. Indeed, although Grès à Voltzia has been studied in detail from the taphonomic, taxonomic and palaeoecological standpoints (Gall 1996, 1972, 1983; Gall & Grauvogel-Stamm 2005), including its aquatic insect forms (e.g. Sinitshenkova *et al.* 2005; Lukashovich *et al.* 2010; Sroka & Staniczek 2022), a detailed analysis of the insect preservation is yet to be carried out.

The evolution of feeding habits in insects is a poorly known subject and largely relies on indirect evidence, such as inferences based on extant relatives, analogous anatomical features (e.g. mouthparts) and ichnofossils (e.g. plant–insect interactions). A few fossils provide direct evidence of feeding habits. These include food sources in association with their potential consumers (e.g. pollen loads clumped to long-proboscid flies; Peñalver *et al.* 2015), coprolites (Richter & Baszio 2001; Peñalver 2002), or preserved gut contents, particularly palynomorphs, in the form of gastroliths (stomach contents) or cololites (intestinal contents) (e.g. Krassilov & Rasnitsyn 1997; Wedmann *et al.* 2021). Fossilized gut contents represent a source of remarkable palaeobiological and palaeoenvironmental data (Seilacher 2002). Although cololites are not rare in arthropods and occur in diverse groups, ages and outcrop types (Knaust 2020), they are easily overlooked or ignored. For instance, they are present in Devonian aquatic isopod relatives preserved as compressions (Robin *et al.* 2021, fig. 1c) and in terrestrial isopods preserved in Cretaceous amber (Sánchez-García *et al.* 2021, figs 7B, 9B). The three-dimensional (3D) cololites preserved in arthropods are usually present in body fossils, and not isolated as more commonly occurs in fossil vertebrates (Seilacher 2002).

Here we provide a detailed analysis of 3D, *in situ* cololites of the most abundant mayfly nymph morphotype from the Spanish earliest Middle Triassic aquatic assemblage of Pedra Alta (Matamales-Andreu *et al.* 2021). The taphonomic study of these mayfly nymphs and their cololites indicates a probable sedimentivorous lifestyle, a

peculiar strategy in insects that could have played an important role during their early colonization of aquatic ecosystems.

GEOGRAPHICAL AND GEOLOGICAL CONTEXT

Mallorca is the largest island of the Balearic Archipelago, located in the western Mediterranean. It was uplifted during the Alpine Orogeny, and it is structurally composed of three main southwest–northeast mountain ranges that range from the Carboniferous to the middle Miocene, surrounded by upper Miocene–Holocene material. A few small and poorly accessible outcrops of Lower–Middle Triassic continental facies, the only ones of Mallorca, appear along the coastal cliffs between Cala d'Estellencs and Punta de son Serralta, at the northwest margin of the Serra de Tramuntana mountains (Fig. 1A). There, four formal lithostratigraphic units have been recently defined (Matamales-Andreu *et al.* 2021), from lower to upper: Punta Roja Formation (Fm.), Estellencs Fm., Pedra Alta Fm. and Son Serralta Fm. (Fig. 1B).

All of the insect fossils considered herein were collected from greenish (sometimes sandy) siltstone beds in the Pedra Alta outcrop (Fig. 1C), which can be included in the upper part of the Estellencs Fm. (Matamales-Andreu *et al.* 2021; Peñalver *et al.* 2022). Although this formation was interpreted to mostly correspond to meandering river and associated floodplain deposits, its upper part consistently possesses a coarser interval that was argued to represent deposits of braided rivers that developed during a period of more energetic water flow (Matamales-Andreu *et al.* 2021). The Pedra Alta palaeoentomological outcrop is part of that interval, and contains a succession consisting of alternating beds of white medium-grained sandstones with tabular cross-bedding, and beds of red or green very finely grained claystones with sub-horizontal lamination, sometimes with intercalated thin horizons of siltstones or very fine-grained sandstones (Fig. 1C). The white sandstones are interpreted as the levée deposits of a braided river, formed during the high-water phases, whereas the siltstones and claystones correspond to the sedimentation of the suspended load when the water table fell and the backswamp was isolated from the main channel (Matamales-Andreu *et al.* 2021; Peñalver *et al.* 2022).

The pools that developed in the backswamp were probably quite persistent in time, perhaps during a whole wet season. Their deepest parts were dysoxic or completely anoxic, as represented by the reduced sediments rich in organic matter (green siltstones), whereas the parts closer to the surface were heavily oxidized (red siltstones) and barely preserve any fossil remains (Matamales-Andreu *et al.* 2021). Such deposits are generally devoid of

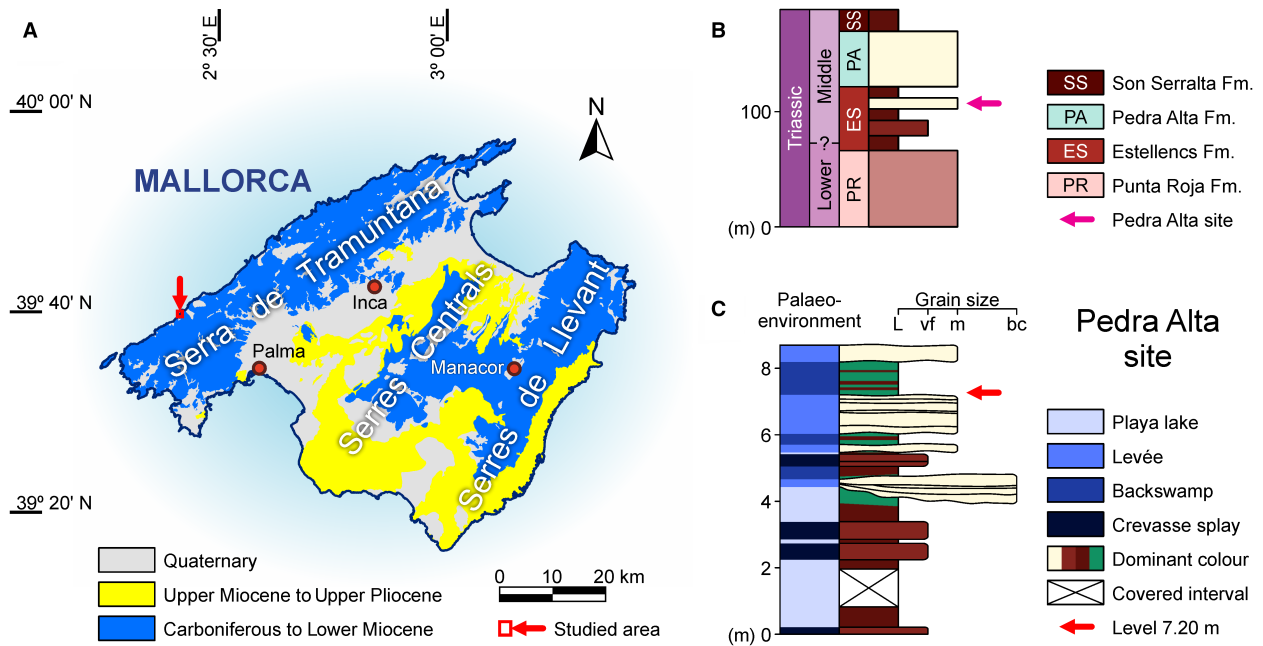


FIG. 1. Geographical and geological context of the Pedra Alta palaeontological site. A, simplified geological map of Mallorca showing the location of the study area. B, stratigraphic framework of the Lower–Middle Triassic continental units of Mallorca, showing the position of the studied section. C, stratigraphic log of the studied section, with the location of the fossiliferous horizons and the four recognized depositional palaeoenvironments (indicated with different shades of blue). *Abbreviations:* bc, intrabasinal microbreccia; L, claystones/siltstone; m, medium-grained sandstone; vf, very fine-grained sandstone. B–C simplified after Matamales-Andreu *et al.* (2021).

bioturbation, although some particular horizons have a local abundance of small, horizontal and vertical burrows. Similar environments to those of the Pedra Alta outcrop were described in the slightly younger locality of Grès à Voltzia of the northern Vosges (France) (e.g. Gall 1972, 1983).

In the upper part of the Estellencs Fm. abundant macrofossils are documented. One example of this is a clam shrimp assemblage that characterizes some of the palaeontological beds, and is distinguished by the presence of valves from *Hornestheria* Kozur & Lepper in Kozur & Weems, 2010 (Scholze & Matamales-Andreu 2021). A single record of an indeterminate fish corresponds to the siltstone beds as well (Matamales-Andreu *et al.* 2021). Finally, a macrofloristic assemblage completes the fossil content of the siltstones from the upper part of the Estellencs Fm., mostly characterized by the presence of branches of the genera *Albertia* Schimper in Voltz, 1837 and *Voltzia* Brongniart, 1828, and other coniferous remains (cones and bracts; Juárez Ruiz & Wachtler 2015).

The Konservat-Lagerstätte of Pedra Alta was most recently dated as Aegean (lower Anisian, Middle Triassic) (Matamales-Andreu *et al.* 2021). Palynomorph analysis pointed to an Anisian age (Calafat 1988). Later, Diez *et al.* (2010) revised the list of species, confirming the attribution to the Anisian and positing that the age could be further constrained in the lower Anisian (= Aegean

substage). Recently, Scholze & Matamales-Andreu (2021) studied the clam shrimp fossils present in the Pedra Alta outcrop and argued that the finding of specimens identified as *Hornestheria* aff. *H. sollingensis* Kozur & Lepper in Kozur & Weems, 2010 (Xiangxiellidae) was indicative of the *H. sollingensis* Zone, characteristic of the uppermost Spathian to Aegean (uppermost Olenekian to lower Anisian). Therefore, both the palynomorphs and the clam shrimps are consistent with an Aegean (lower Anisian) age for this outcrop. This contrasts with the similar palaeoenvironments of the Grès à Voltzia (Gall & Grauvogel-Stamm 2005; Matamales-Andreu *et al.* 2021), which have been attributed to the Bithynian substage (middle Anisian) based on its clam shrimp fauna (*E. albertii albertii* Zone of Kozur & Weems 2010). This means that Pedra Alta is up to c. 2 myr older than Grès à Voltzia.

MATERIAL AND METHOD

The more than 50 specimens included in this study come from the Pedra Alta section (at 4.75 m and 7.20–7.30 m), in the upper part of the Estellencs Fm. (Mallorca, Balearic Islands, Spain). These specimens are present in two collections, one public and the other a historical private collection. Field sampling was carried out under the excavation permits from the Comissió Insular de Patrimoni Històric

of the Consell de Mallorca with reference codes 305/2019 and 52/2021. The specimens collected during this palaeontological excavation, and the samples prepared from them for the present study, are part of the public collection and are housed at Museu de Mallorca (Palma, Mallorca, Balearic Islands, Spain) with an abbreviation in the form: DA21/[campaign number]-[horizon number]-[specimen number]. All figured specimens in this study belong to this collection.

The fossilized exoskeletons studied herein are better visualized using low-angle light due to the contrast of their shiny surface with the matt sheen of the rock matrix. The cololites have been observed in preparations under optic microscopy, namely by transparency using transmitted light. These preparations were made as follows. Small sections (S1–S7) of the 3D cololites were easily obtained by pressing with a pin on one side and were prepared on microscope slides using glue (instant cyanoacrylate adhesive; Ethyl-cyanoacrylate, LOCTITE®, Henkel Adhesives) (Fig. S1); two additional samples of the rock matrix, in association with cololites but outside the body perimeter, were obtained for comparison (Sa1 matrix and Sa2 matrix). All of the sections were prepared in longitudinal position except S5 and one of the two portions obtained from S7. The contents of the cololite sample in the glue drop were strongly contrasted and enhanced, being semitransparent under the optic microscope. The glue drop needed several minutes to harden, allowing time to add the cololite sample and to take photographs and/or camera lucida drawings. When the cololite hardened, the glue could be cut easily using a scalpel under the stereomicroscope, exposing the core of the cololite and obtaining a thin preparation of the sample to be observed using scanning electron microscopy (SEM), including serial transversal sections while avoiding sampling deformation due to the glue consistency. A similar methodology was conducted for the ‘Sa2 matrix’ sample, whereas the ‘Sa1 matrix’ sample was obtained and mounted directly on the SEM sample holder (Fig. S2).

SEM images of a cololite/digestive tube and a rock matrix sample were taken using a HITACHI Tabletop Microscope TM4000Plus, without gold sputtering; and element analysis (energy dispersive x-ray spectroscopy: EDS) of a cololite section fragment and a rock matrix sample were obtained using a Hitachi S4800 Electronic Microscope, both at the Microscopy Services (SCSIE) of the University of Valencia (Spain). Margarite pseudomorphs after chiastolite have been studied using a JEOL JSM-6010 Plus scanning electron microscope equipped with an energy-dispersive x-ray microanalyser and a back-scattered electron detector (BSE) at the laboratories of the Instituto Geológico y Minero de España (IGME, Madrid), CSIC. The photographs of complete slab surfaces were taken using a Canon EOS 40D digital camera. Some photographs were taken with a Canon EOS 650D digital camera using

Macrofotografía version 1.1.0.5 (IGME-CSIC, Madrid, Spain), which integrates the Helicon Focus software to create composite photographs by integrating sequential images obtained at different focal planes. Microphotographs were taken with a ColorView IIIu Soft Imaging System digital camera attached to an Olympus BX51 microscope (IGME-CSIC, Madrid, Spain). An Olympus BX53 compound microscope equipped with an attached camera lucida tube was used to make the drawings of the fossil insects and their gut contents (IGME-CSIC, Valencia). The non-prepared specimens were examined, drawn and photographed dried and/or wet with a mixture of alcohol and water (the alcohol–water mixture enhances the contrast of the carbonaceous film against the matrix). Photography was enhanced in Photoshop CS2 version 9.0 (<https://www.adobe.com>) for increased contrast, and composite figures were prepared using the same software.

RESULTS

The mayfly nymphal morphotype bearing the cololites analysed herein occurs in Pedra Alta under diverse developmental stages, with body lengths ranging from *c.* 3–4 mm to 6.6–7.0 mm. These nymphs constitute the most abundant insect record in some beds, including slabs indicative of mass records (Fig. 2). All of the specimens are articulated and show differential preservation. The exoskeleton is generally preserved as a transparent film imprinting a fine relief in the rock matrix. Some external areas are fossilized as darker (originally more sclerotized) carbonaceous films, such as the cephalic capsule and the mouthparts. However, the most evident structures in the fossil nymphs are generally the gut tubes, either directly due to their sclerotization or indirectly due to the general presence of cololites inside them (Figs 3, 4D, E). Details of the foregut can be distinguished in one particularly well-preserved specimen as darker structures, showing an originally greater degree of sclerotization by the presence of cuticle due to the ectodermal origin of the foregut (Chapman 2013; Holtorf *et al.* 2019); visible parts include the pharynx and proventriculus, and the anterior part of the oesophagus (Fig. 4C). The remaining parts of the gut in these nymphs are also visible, indicating a differential resistance to decay. In a transversal cut of a cololite section covered by the gut epithelium under SEM, the epithelium is preserved as a carbonaceous film without cuticle layers (Fig. 5), which indicates that this section is most likely to have belonged to the midgut. In any case, the limits between the fore-, mid- and hind gut are not evident in these fossils. These nymphs can be accurately attributed to Ephemeroptera because of the typical three apical abdominal appendages. A general description of this mayfly nymph morphotype is provided by Matamales-Andreu *et al.* (2021).

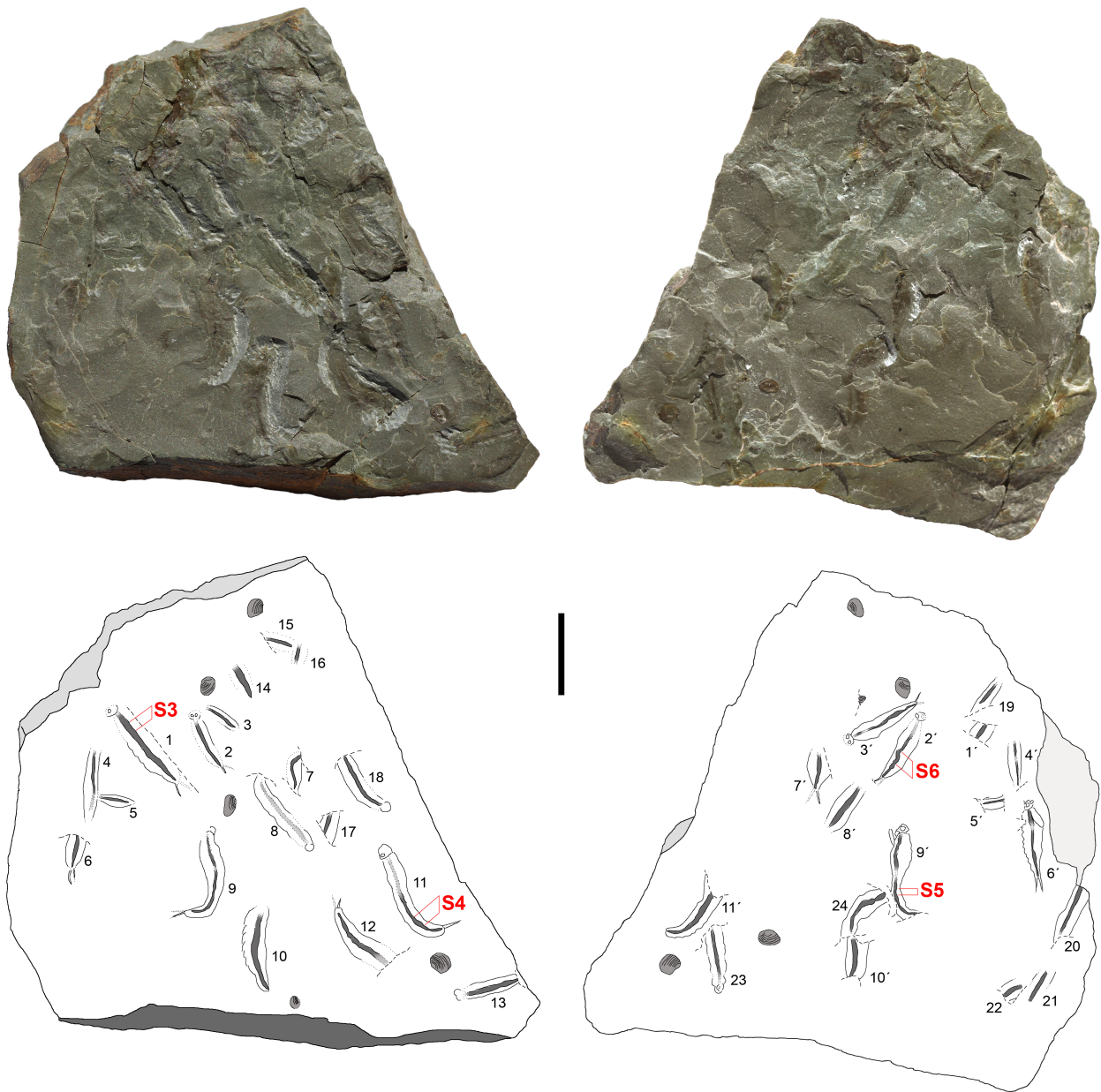


FIG. 2. Part (left) and counterpart of slab DA21/14-02-01 showing a mass record of the mayfly nymph morphotype with cololitic content from Pedra Alta (24 specimens) and six specimens of clam shrimp *Hornestheria* aff. *H. sollingensis*. Not all of the specimens are in the same surface (note that parts and counterparts share the same numbers). Digestive guts are represented in grey colour and without distinction of their cololite portions (see the detailed drawings of selected guts in Fig. 6). S3–S6 indicate sections of cololites removed and prepared. Scale bar represents 1 cm.

The majority of the mayfly nymphs from Pedra Alta have gut contents, which are preserved in three dimensions and *in situ*. These cololites are so conspicuous that nymphs were often detected in the field because of these structures. The cololites are easily detachable, more commonly during rock exfoliation. They are sometimes split into part and counterpart. Detached cololites leave a conspicuous mark as a negative relief (Fig. 3). The width of

the cololites depends on the stage of nymph development, ranging from 0.1 to 0.6 mm, with an average of 0.48 mm ($n = 20$) (Fig. 4). The cololites are not homogeneous along their lengths, showing constrictions and/or different regions separated by short sections of gut tube that were apparently empty (Fig. 6). Moreover, the cololites are present in the midgut and the hind gut, and are practically absent in the foregut (Fig. 6).

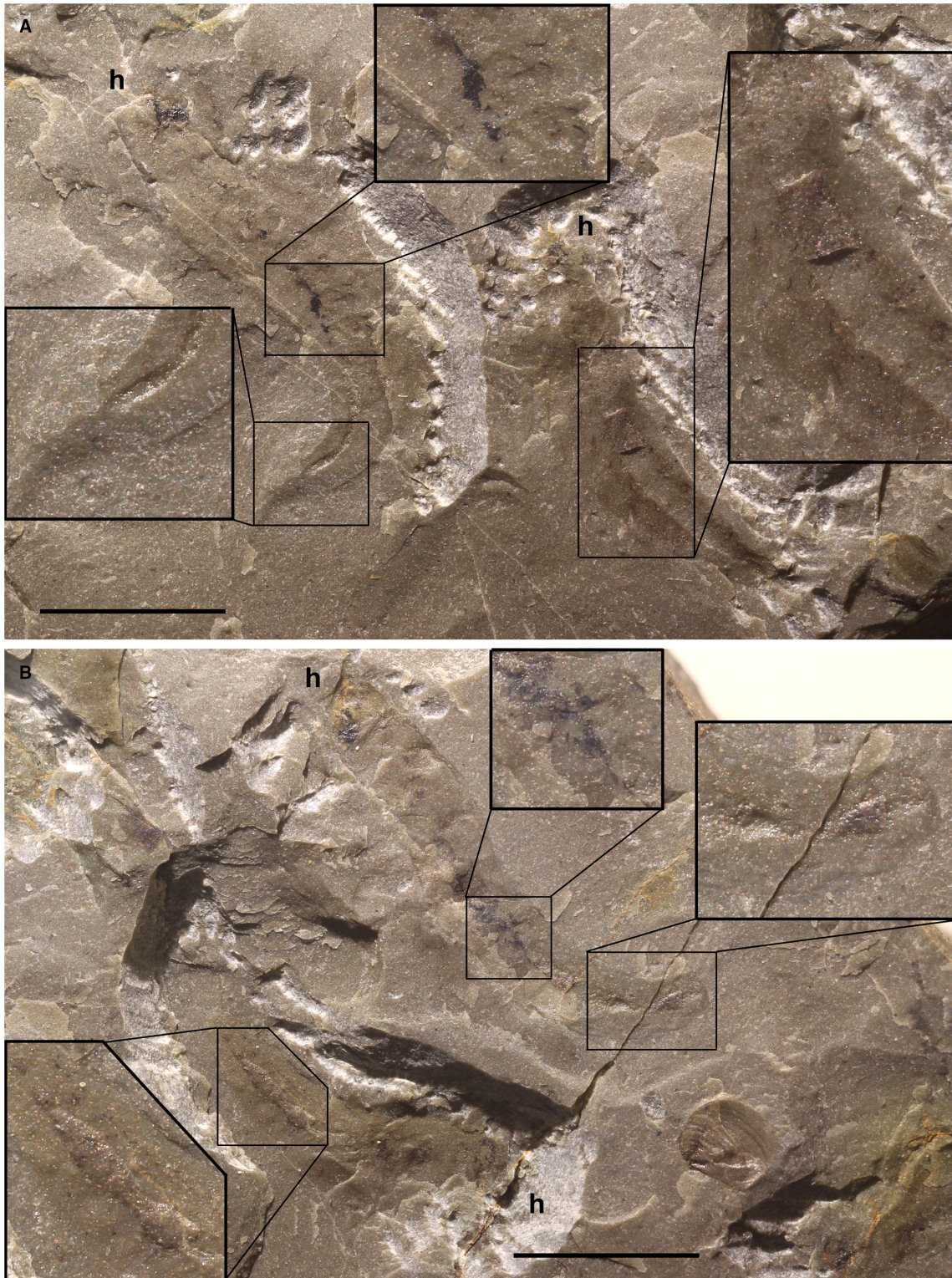


FIG. 3. Detail of four mayfly nymphs from the part of mass record slab DA21/14-02-01 in Fig. 2. A, specimens 9 (left) and 10 (right): details of the negative relief left in the hind gut by a lost 3D cololite (left inset), an undetached cololite section in midgut (right inset), and the carbonaceous film preserved of a gut section in midgut (top inset). B, specimens 12 (left) and 11 (right) showing similar details as in (A); note the clam shrimp *Hornestheria* aff. *H. sollingensis* at the right. Abbreviation: h, head or anterior part of the body. Images created from consecutive pictures taken at successive focal planes. Scale bars represent 5 mm.

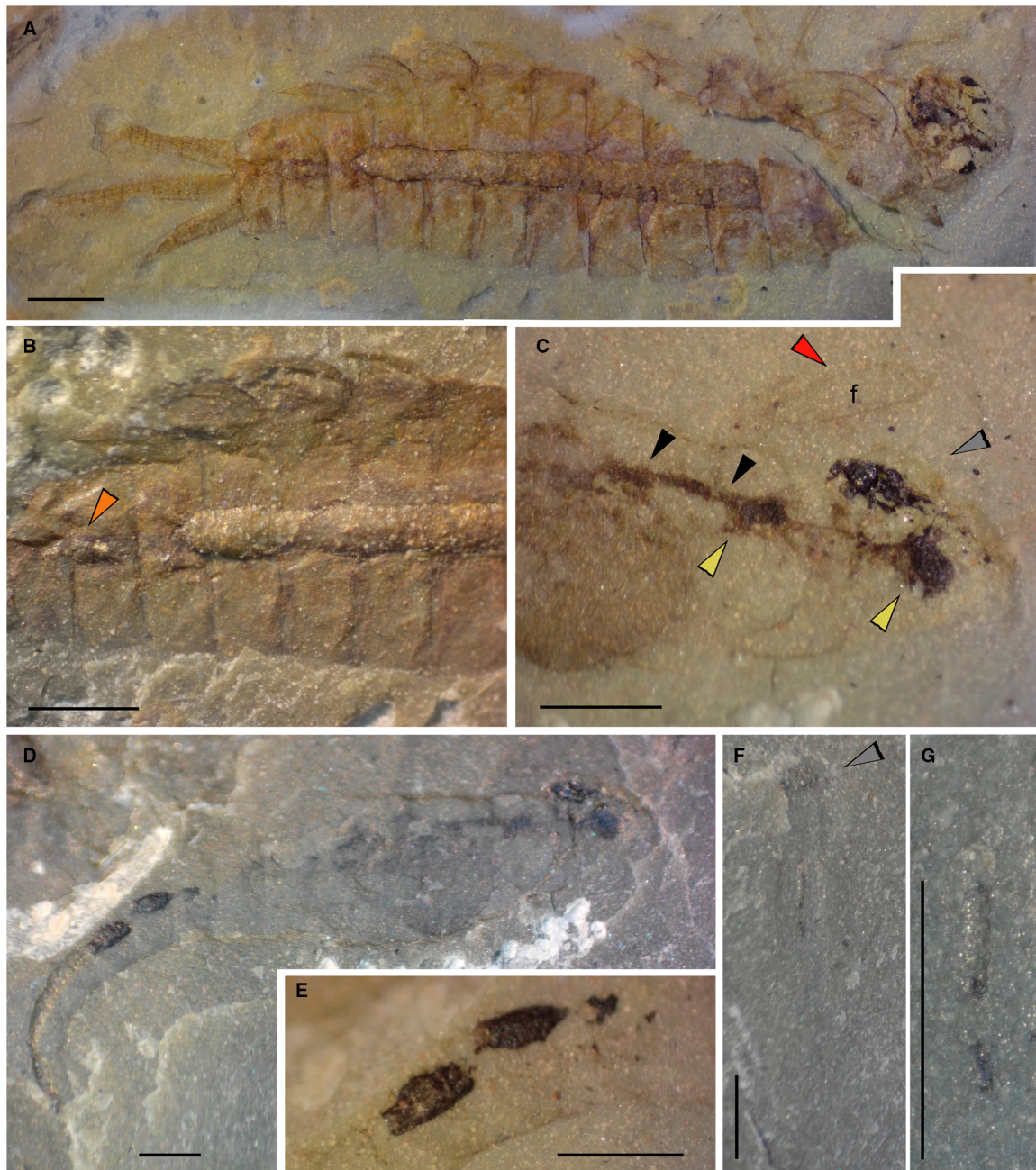


FIG. 4. Digestive guts of selected virtually complete mayfly nymphs from Pedra Alta, showing diverse typologies of 3D cololites and anatomical details of the foregut. A, specimen DA21/03-02-19 showing a massive cololite, virtually infilling its gut. B, detail of the same specimen showing the terminal section of the cololite and a small terminal cololite part in the rectum or nearby (orange arrowhead). C, detail of the head (grey arrowhead) of specimen 9' in DA21/14-02-01 (Fig. 2) with an anterior dark mass corresponding to the mouthparts and the carbonaceous foregut showing the pharynx (the anterior yellow arrowhead most likely indicates the pharynx and the posterior indicates the proventriculus) and the anterior part of the oesophagus (black arrowheads) (red arrowhead indicates the swollen femur of the foreleg). D, habitus of the same specimen showing a cololite in several portions as a combination of the 3D content and the carbonaceous epithelium of the gut covering it. E, detail of the cololite parts of the same specimen (Fig. 5; Fig. S1). F, minute specimen, DA21/03-02-05, showing a small cololite in two parts along one-third of the body length, as an example of poorly preserved small mayfly nymphs that are identifiable only due to their preserved cololite; G, detail of the small cololite of the same specimen. Habitus of specimen in A, head in C, and cololite in E were photographed wet using a mixture of alcohol and water; the rest were photographed dry. *Abbreviation:* f, fore femur. Scale bars represent 1 mm.

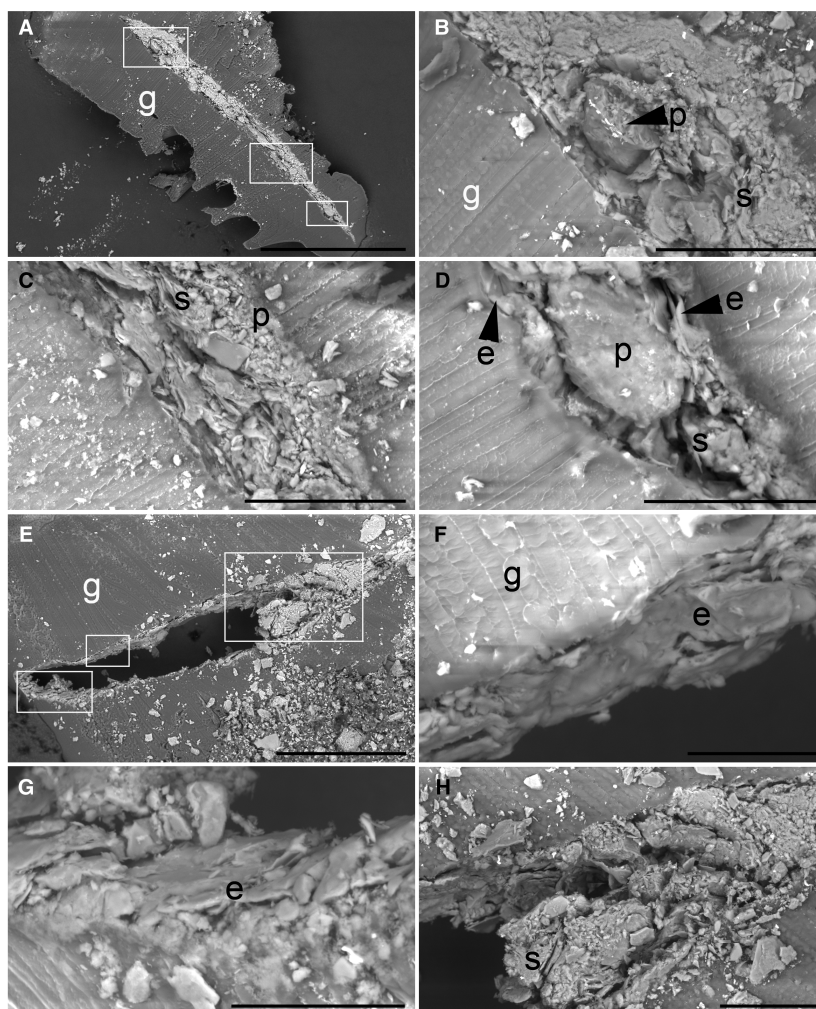
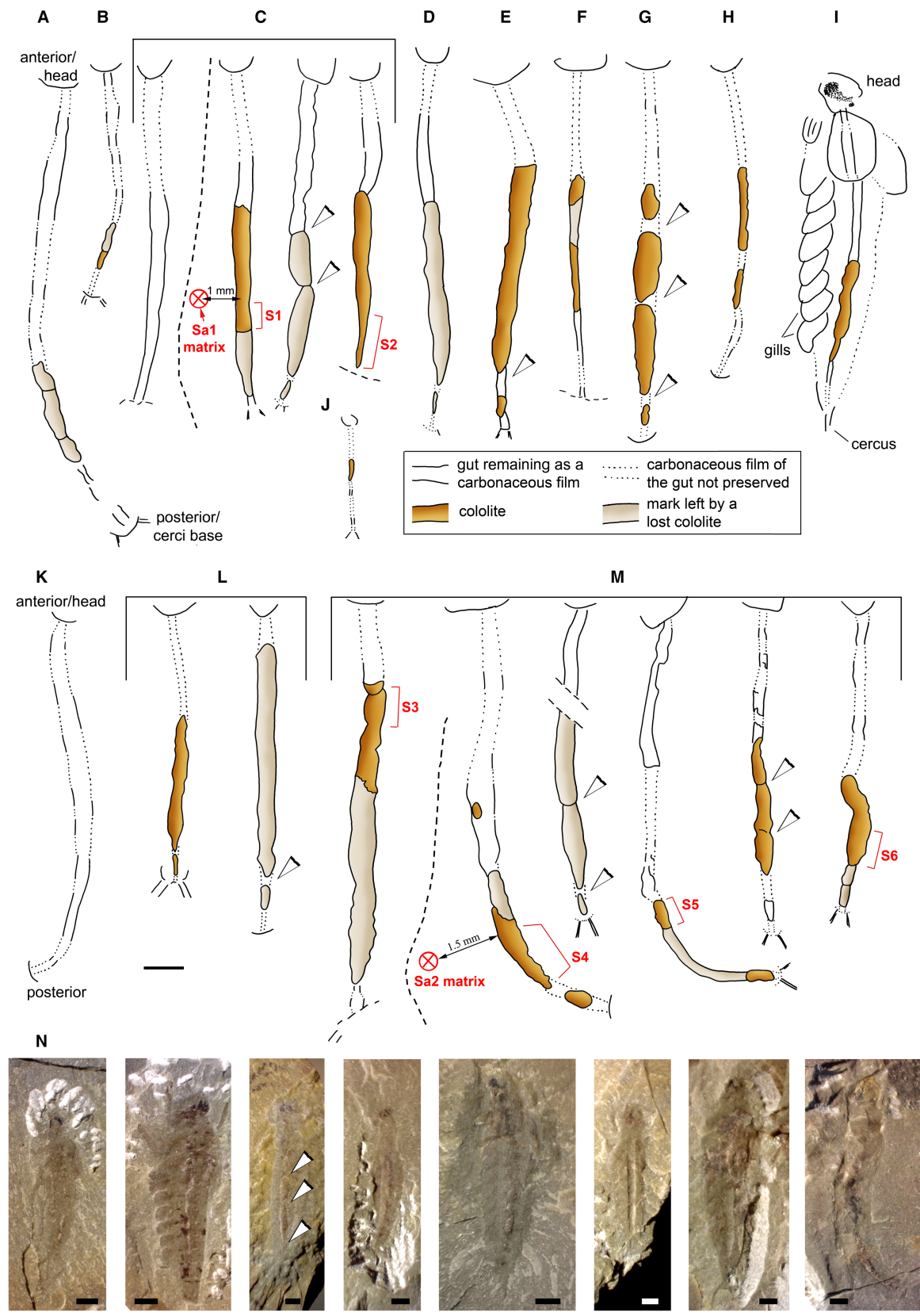


FIG. 5. SEM images of the digestive tube and its content (cololite) of specimen 9' in DA21/14-02-01, in two transversal cuts from a preparation using instant glue to externally fix the structures (Fig. S1). A, E, general views of two cuts; B–D, details from A; F–H, details from E. A, cut 1 showing a strongly compacted sectioned sample (13:1 compaction ratio); B–C, details of gut content showing large particles (sedimentary particles or compacted sedimentary material not exfoliated by the scalpel) and squamous parts; D, gut tube epithelium preserved as a carbonaceous film. E, cut 2 with loss of cololite content during cutting (given that the glue did not harden the sample, obtaining the compaction ratio was not possible); F–G, details of gut tube epithelium preserved as a carbonaceous film in direct contact with the glue that fixed the cololite sectioned sample; H, gut content or cololite, mostly with a squamous aspect. *Abbreviations:* e, epithelium of the digestive tube, most likely from the midgut; g, glue, p, particle (non-squamous); s, squamous digestive content. Scale bars represent: 300 μm (A); 50 μm (B, C, H); 30 μm (D, G); 200 μm (E); 20 μm (F).

The cololites are composed of inorganic and organic detrital particles as well as small aggregates. The inorganic particles are the main components, and consist mostly of very

fine-grained claystone, a few conspicuous sand grains of varied size, and abundant mineral crystals with a dark nucleus, 35 μm in average dimension ($n = 40$ from specimen 11 in

FIG. 6. Digestive guts of selected virtually complete mayfly nymphs from Pedra Alta. Camera lucida drawings of 22 specimens indicating the 3D cololite sections (S1–S6) and rock matrix samples (Sa1 matrix–Sa2 matrix) obtained and prepared (to the same scale); only the gut has been drawn, with its boundaries schematically drafted (head position and base of cerci), except for specimen (I), in which other anatomical features have been drawn. Specimens grouped together correspond to specimens present in the same rock slab. A, DA21/03-02-109. B, DA21/03-02-97. C, DA21/03-02-20. D, DA21/03-02-45. E, DA21/03-02-19. F, DA21/03-02-89. G, DA21/03-02-98. H, DA21/03-02-87. I, DA21/03-02-23. J, DA21/03-02-05. K, DA21/03-02-117. L, DA21/03-02-90. M, DA21/14-02-01 (specimens 1, 11, 6', 9', 3' and 2', respectively, in Fig. 2). N, some of the drawn specimens (not to the same scale). White arrowheads indicate separation of gut content between different stages of intake in some selected specimens. Scale bars represent 1 mm.



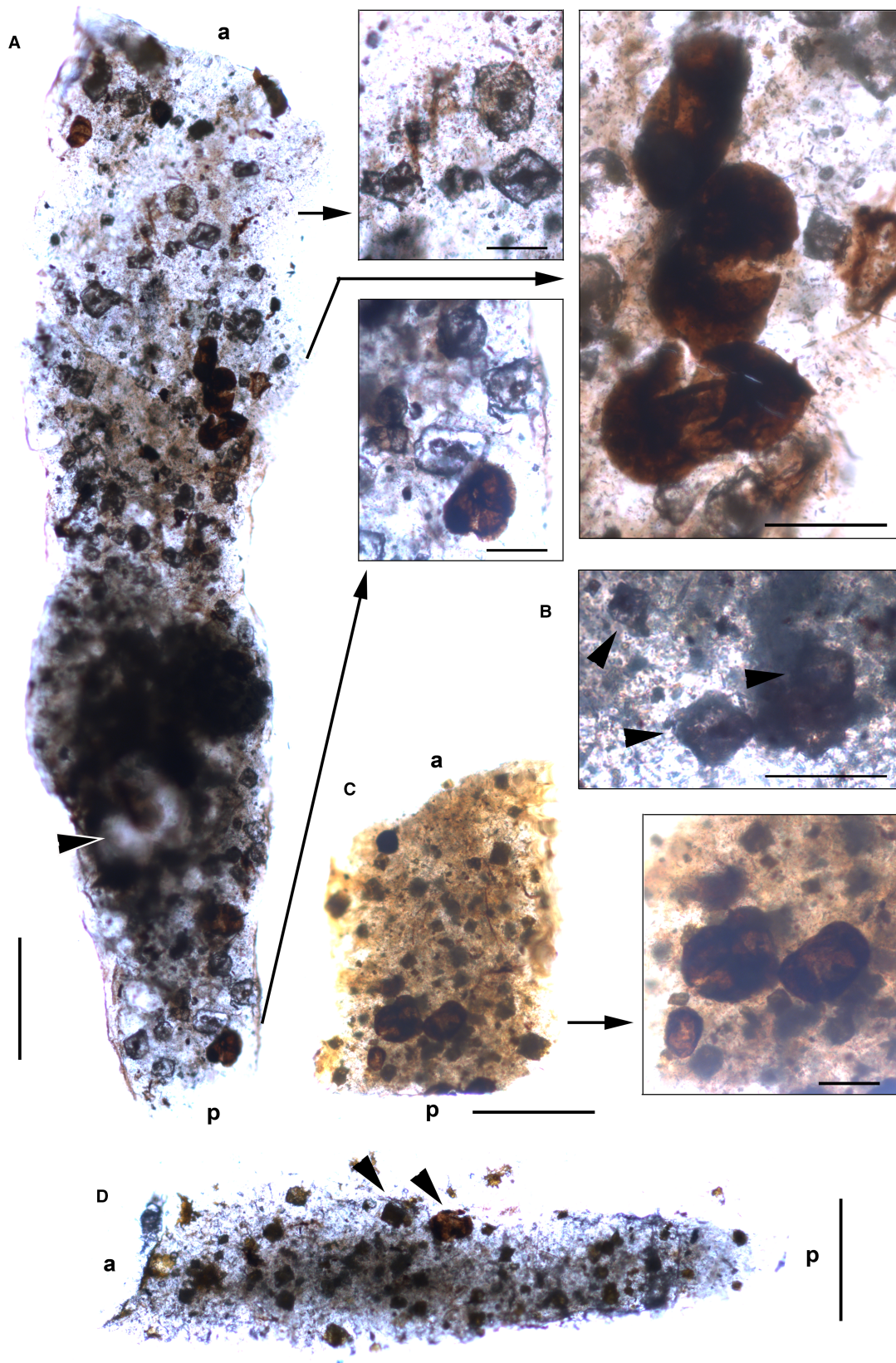


FIG. 7. Preparations of three representative 3D cololite sections and one rock matrix sample. A, long portion of a cololite from specimen 11 in DA21/14-02-01 (section S4, Fig. 2; Fig. S3) and details of the margarite pseudomorphs and refractive organic matter, bisaccate pollen grains of cf. *Alisporites* sp. and/or cf. *Lunatisporites* sp. being the only organic remains that could be identified; arrow-head indicates a relatively large sand grain (out of focus in this microphotograph). B, margarite pseudomorphs after chiastolite (arrow-heads point to three of them) in a sample from the nymph-bearing rock. C, short cololite portion from a specimen in slab DA21/03-02-20 (section S1; Fig. 6) showing its content in refractive organic matter including pollen grains of cf. *Alisporites* sp. and/or cf. *Lunatisporites* sp. D, long portion of a small cololite sampled from another specimen in slab DA21/03-02-20 (section S2; Fig. 6; Fig. S1); arrowheads indicate a margarite pseudomorph (left) and a well-preserved pollen grain (right). *Abbreviations:* a, anterior end; p, posterior end. Scale bars represent: 0.2 mm (A–D); 0.05 mm (insets from A and C).

DA21/14-02-01, Fig. 7A). The organic detrital particles represent a low percentage of the total cololite content, and include: (1) very particulate debris, most likely to be due to plant decomposition/fragmentation and thus cellulosic in nature; (2) conspicuous bisaccate pollen grains, up to c. 90 μm long, of cf. *Alisporites* sp. and/or cf. *Lunatisporites* sp. (E. Barrón, pers. comm. 2021; Diez 2000; Diez *et al.* 2010); the type of preparations and the suboptimal preservation of the pollen grains prevent a confident identification (Fig. 7; Fig. S3); and (3) small aggregates. Last, the cololites also contain a few small indeterminate tubular structures, suggestive of filamentous microbial remains but which cannot be identified with confidence. Under SEM, the few areas observed of cololite content samples were very homogeneous in appearance, and sand grains, mineral crystals or organic detritus were not evident (Fig. 5; Fig. S2). Although the cololites were flattened due to the compaction of the host sediment, it was less intense than that of the host bodies. This is evident in the cololite section that was prepared for observation of its transversal section ($650 \times 50 \mu\text{m}$) to avoid sample deformation (Fig. 5; Fig. S1).

The nymph-bearing rock is composed of the same constituents as the cololites: very fine-grained inorganic detrital particles, crystals with a dark nucleus, and organic components such as conspicuous bisaccate pollen grains (Fig. 7; Fig. S3). EDS analyses (in areas without the crystals described above) indicated the same elemental composition, with a predominance of Si, Al and O (Fig. 8). Removed cololite sections showed the same squamous breaking parallel to the slab plane as the nymph-bearing rock when sampled (Fig. 5; Fig. S2).

DISCUSSION

Cololite origin and composition

The mayfly nymphs bearing cololites studied herein are remarkably abundant, in some cases fossilized in mass records (Fig. 2). An extensive record of transported lotic insects in continental compression deposits, where burial under suitable preservational conditions typically occurs, would be implausible, given the taphonomic processes that

usually occur in these deposits. The extraordinary abundance of the present mayfly nymphs strongly suggests that these aquatic insects lived in the standing waters in which the sediment was deposited and, thus, that they underwent minimal transport (para-autochthony).

The pervasiveness of cololites in the nymph morphotype addressed herein and their preservation, generally present beyond the anterior third of the gut length, and sometimes still present in the rectum or nearby (Figs 4A, 6), are evidence of food intake. Thus, an accidental and abundant ingestion of sediment due to the physical external forces in an early biostratinomic phase conducive to the death and burial of these nymphs can be ruled out. Further evidence is provided by the fact that most of the cololites are composed of several sections, both in consecutive contact in the gut or with gaps between them, indicating different intake stages (Fig. 6A, C, E, G, H, L, M). The shared appearance and similar composition, consistent with that of claystone (constituted by phyllosilicates) (Fig. 8), of the rock matrix and the cololites, show that the mayfly nymphs ingested virtually the same sediment that buried their carcasses but in an oxygenated environment while alive, further indicating para-autochthony for this mayfly nymph assemblage.

The crystals with a dark nucleus that are present in both the cololites and the rock matrix (Fig. S3) are margarite pseudomorphs after chiastolite (andalusite). The presence of Si, Al and Ca in the crystals and of C in the nucleus obtained with EDS analysis indicates that this phyllosilicate occurs as a pseudomorphic replacement of previous chiastolite (Guidotti *et al.* 1979). Thus, the margarite pseudomorphs are diagenetic rather than representing detrital particles in the original sediment ingested. Due to the similar composition of the cololites and the rock matrix, it is expected that the same effects of diagenesis would be observed. In organic matter-rich fossiliferous claystone, chiastolite is formed by low-grade thermal metamorphism. Probably, this was due to the intrusion of volcanic or sub-volcanic rocks in Permian–Triassic rocks of nearby areas of the Serra de Tramuntana (Enrique 1986), during Late Triassic magmatic episodes (Sanz *et al.* 2013).

Food processing in the gut must have been fast, given that the sediment that originated the claystone was poor

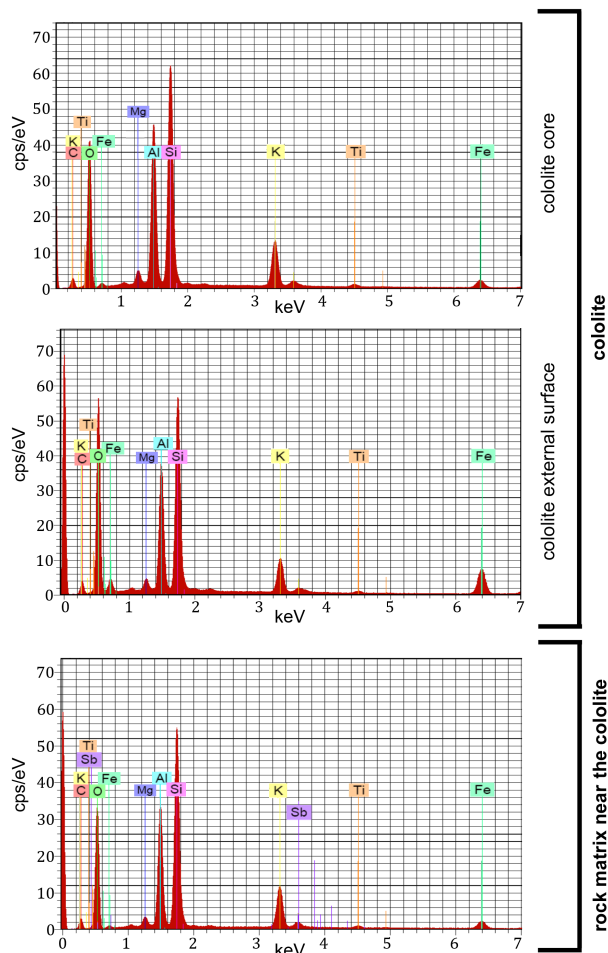


FIG. 8. Elemental analyses of sub-square areas with sides *c.* 0.1×0.05 mm of the cololite (sample from section S1) and rock matrix surfaces, of a specimen in slab DA21/03-02-20 (specimen drawn in Fig. 6C), for comparison (Fig. S2). The top and middle panels show the analyses of the core of the cololite, cut using a surgical scalpel, and its external surface (both of the same sample). The bottom panel shows the analysis of a fresh surface of a flake (Sa1 matrix; Fig. 6) from the rock matrix 1 mm from the removed cololite section (S1). Note the elemental similitude between the two types of materials and that the analysed areas did not contain margarite pseudomorphs.

in organic matter. This could explain the lack of cololite remains in some studied nymph specimens (e.g. Fig. 6K). In any case, the available specimens show that the sediment transit towards the midgut was probably fast. In some extant mayfly nymphs such as *Baetis* Leach, 1815 or *Cloeon* Leach, 1815 species, it takes only *c.* 30 min for the ingested matter to pass through the gut (Sartori & Brittain 2015).

Seilacher (2002) showed that coprolites and cololites usually retain their original three-dimensionality and so they are partly resistant to flattening. This also occurs in

deposits where other organic remains, such as ammonite shells, are strongly flattened. However, that is not always the case: abundant coprolites in the Iberian Peninsula Miocene lacustrine deposits of the Bicornb-Quesa and Rubielos de Mora basins (oil-shales) and the Ribesalbes-Alcora Basin (laminated dolomicrites), produced by salamanders (in the latter two) or by fishes (Peñalver 2002; Peñalver & Gaudant 2010; Peñalver *et al.* 2016), are strongly flattened as carbonaceous films. The 3D cololites studied herein are different from those discussed by Seilacher (2002), in that slight flattening has occurred and they are preserved *in situ*.

The exceptional preservation of the cololites in Pedra Alta and the absence of complete flattening produced by both lithostatic pressure and decomposition can be explained by: (1) the very same nature of the original very fine-grained clay-dominated sediment both in the gut content and in the host matrix; (2) the high sediment content in some parts of the gut due to ingestion, and the differential resistance of the gut content (ingested incompressible particles or sediment) with regards to the nymphal body when subjected to compaction; and (3) the similar early processes of mineralization in the gut sediment and the surrounding sediment of burial. An early mineralization of the cololites induced by bacteria seems not to have occurred or, at least, it was not the main cause of their three-dimensionality. The similar squamous breaking of the cololites and the rock matrix suggests similar processes of compaction during diagenesis.

Considering that the cololite of specimen 9' in the counterpart of the slab DA21/14-02-01 was originally circular in transversal section when infilled, and that fossil diagenetic flattening did not alter the lateral dimensions of the whole original carcass in the position of burial, based on the expected or 'natural' dimensions of this and the other mayfly nymphs, the compaction ratio of the sediment as gut content can be calculated. The transversal preparation of the cololite section from specimen 9' is shown in Figure 5A and Figure S1C (see Fig. 4D, E for the cololite section after removal). The current dimensions of the transversal section imply a *c.* 13:1 compaction ratio, but apparently, it was slightly lower in the case of cololite sections from guts that are more infilled by sediment, for instance in the specimen DA21/03-02-19 (Fig. 4A), in contrast to sections of small isolated cololite portions due to poor sediment intake. This compaction ratio is consistent with that generally found in fine-grained muds (Whittington 1978). The compaction ratio for the rest of the body was extreme, considering the typical preservation as a carbonaceous film in compression fossils when early intense mineralization processes are absent (Peñalver *et al.* 1996).

Three-dimensional structures similar to the cololites herein reported have been observed in some Cambrian arthropods from the Burgess Shale and Chengjiang localities. These were first interpreted as guts infilled by mud in sedimentivorous forms (e.g. in the paracrustacean *Canadaspis laevigata* (Hou & Bergström, 1991); see Hou & Bergström 1997), but later were deemed as serially repeated midgut glands preferentially permineralized by early diagenetic phosphate (Butterfield 2002a, 2002b; also see Vannier *et al.* 2014). Some of the photographs in Hou & Bergström (1997) show 3D gut tubes very similar to mud cololites, instead of the clear 3D glands also figured in that work and others. Vannier & Chen (2002) also considered that these 3D gut tubes in naraoiids apparently infilled with mud are taphonomic artefacts, and also noted the possibility of accidental mud ingestion by individuals trapped alive in turbiditic flows. As Butterfield (2002a) and Hou & Bergström (1997) noted, the gut contents of these fossils were not analysed in detail to clarify their nature and origin. In contrast, Vannier *et al.* (2014, fig. 1a) presented a lobopodian from the Chengjiang biota with its seriate 3D glands flanking a 3D cololite located in the main part of the gut, but the analysis of its content was not detailed or conclusive.

Detritivorous lifestyle of the mayfly nymphs

Extant mayfly nymphs (adults do not feed) are usually scrapers or collectors (Cummins 1973; Sartori & Brittain 2015). Based on feeding mechanism, Cummins (1973) classified collectors into filter or suspension feeders and sediment or deposit (surface) feeders, the latter considered as fine particle detritivores (decomposing organic matter) according to the type of dominant food ingested. The same author defined detritivory as ‘the intake of nonliving particulate organic matter and the nonphotosynthetic microorganisms that are always associated with it (detritus)’. The mayfly nymph morphotype bearing the cololites reported herein was recently described as a ‘detritivore nymph type’ (Matamales-Andreu *et al.* 2021) following the preliminary optic assessment of the cololites, which showed that most of their content was composed of inorganic particles and that organic detritus was present only in very small amounts. The detailed analysis of the cololites herein provides an insight into the specific feeding habits of the Triassic freshwater mayfly nymphs containing them.

The nymphs of the extant burrowing mayfly *Hexagenia limbata* (Serville, 1829) feed using two strategies, which have been repeatedly observed in the field and in experimental conditions (Hunt 1953; Zimmerman & Wissing 1980; Dermott 1981). First, they create a water flux inside their burrows by movements of their abdominal

respiratory gills, which brings the organic and inorganic particles suspended in the water (seston) to their mouthparts for processing. Second, they feed directly on the sediment, at times on macroscopic particles such as plant fragments, that is present at their burrow entrances with the aid of their forelegs. *Hexagenia limbata* nymphs feed continuously during the day and night and ingest over 100% of their dry body weight per day (Zimmerman & Wissing 1980; Sartori & Brittain 2015), sometimes reaching 192% (Dermott 1981). Research on the ingestion rate of this species suggested that its main food source is the sediment directly consumed rather than the seston brought into the burrows by the gill movements (Dermott 1981; Bachteram *et al.* 2005). This could also be the case for other *Hexagenia* species, given that 90% of the gut contents of *H. munda* Eaton, 1883 consisted of sand and clay (Walker 1970; Zimmerman & Wissing 1980).

The high quantity of original sediment in the cololites studied herein suggests that the Triassic mayfly nymphs largely fed on selected portions of deposited sediment directly, together with (or instead of) other strategies to obtain organic matter, such as suspension feeding through the active production of currents, implying the concomitant ingestion of abundant suspended detrital clays. The presence of several sand grains in the cololite from specimen 11 in DA21/14-02-01 (Fig. 7A; Fig. S3), several orders of magnitude larger in size than the clay particles, supports the hypothesis of the ingestion of selected portions of benthic sediment. Furthermore, a richer content of organic matter particles in the cololites than in the nymph-bearing rock, particularly evident in the differential abundance of pollen grains, suggests selection of sediment rich in organic matter and/or a different concentration of organic matter in the oxygenated areas (shallow, sublittoral areas) in respect to the dysoxic or completely anoxic areas of sedimentation and burial. Aside from the fine particulate organic matter and small remains such as pollen grains present in the sediment, the ingestion of mud would have also entailed feeding on the microbial community in the sediment. Bacteria, protozoans, fungi and algae present in the sediment play an important role in the feeding of the extant *Hexagenia limbata* nymphs (Zimmerman & Wissing 1980).

Although *Hexagenia* species such as *H. limbata* and *H. munda* could represent plausible extant ecological analogues of the Pedra Alta Triassic mayfly nymphs regarding their feeding habits, there is no evidence of typical mayfly burrows in the Pedra Alta locality, unlike in Grès à Voltzia, where U-shaped burrows tentatively attributed to ephemeroïd mayflies are present (Sinitschenkova 2002, 2003). Although the structure of the forelegs of the nymph morphotype studied herein may be compatible with that of a burrowing detritivore (Fig. 4C), it is also possible that the legs were simply used to obtain selected

small portions for ingestion as occurs in *Hexagenia*. Given that the Pedra Alta detritivorous mayfly nymphs do not preserve mouthpart details and their body preservation is poor (Figs 3, 4C), a filter-feeding habit in burrows as occurs in extant *Hexagenia* species, and perhaps complementary to mud-eating, can be neither ascertained nor ruled out.

Single instances of other mayfly nymph taxa (Sinitshenkova *et al.* 2005) (*Triassoephemera punctata* Sinitshenkova & Marchal-Papier in Sinitshenkova *et al.*, 2005 (Triassoephemeridae), *Triassonurus doliiformis* Sinitshenkova & Marchal-Papier in Sinitshenkova *et al.*, 2005 (Siphonuridae) and cf. *Voltziaephemera fossoria* Sinitshenkova & Marchal-Papier in Sinitshenkova *et al.*, 2005 (Voltziaephemeridae)) have been found in Pedra Alta, but they all lack guts with mud infilling (Matamales-Andreu *et al.* 2021). Although these taxa are also present in the slightly younger lower Middle Triassic Grès à Voltzia locality, according to the published descriptions and the figured specimens, none of these mayfly taxa or others from this locality has cololite content (Sinitshenkova *et al.* 2005). Differences in the nature of the pool sediments was not likely to be the cause of the absence of gut content in the mayfly nymphs, considering that the insects in Grès à Voltzia have also been found in very fine-grained laminated clay (Briggs & Gall 1990) and are mostly articulated, indicating limited biostratinomic transport, similar to the case of Pedra Alta. Two species from Grès à Voltzia, *Mesoplectopteron longipes* Handlirsch, 1918 and *Vogesonympa ludovici* Sinitshenkova & Papier in Sinitshenkova *et al.*, 2005, were recently regarded as passive filter feeders by Sroka & Staniczek (2022), based on their double row of pronounced setae along the prothoracic leg and their dense mouthpart setation. Details enabling the determination of feeding habits with confidence have not been described from other Grès à Voltzia mayfly nymphs, but they probably were detritus collectors or algal feeders, while burrowing forms could filter detritus (Sinitshenkova *et al.* 2005). Sinitshenkova (2000) considered that one of the specimens from Pedra Alta, which had been previously reported and figured by Calafat (1988), was a burrowing form. Later, the same author suggested that this morphotype from Pedra Alta had a filter-feeding habit (Sinitshenkova 2002), as was also presumed for the burrowing taxa of Grès à Voltzia (Sinitshenkova *et al.* 2005). Note that the specimen from the Pedra Alta outcrop discussed by Sinitshenkova (2000, 2002) was tentatively identified by Matamales-Andreu *et al.* (2021) as *Voltziaephemera fossoria*. The current knowledge indicates that the detritivorous nymph morphotype from Pedra Alta studied herein was probably absent in the Grès à Voltzia aquatic palaeoenvironment. Exuviae belonging to this morphotype have not been found in Pedra Alta, which is probably related to their lower preservation potential.

Early detritivory in insects of aquatic environments

There is evidence of Palaeozoic detritivory in groups such as myriapods and arachnids (mites) (e.g. Falcon-Lang *et al.* 2015). Among the Hexapoda, the Collembola and other so-called ‘apterygotes’ were probably detritivorous since the Devonian (Labandeira 2019). It is highly probable that detritivory also occurred in some Palaeozoic adults or preimaginal stages of winged insects, but the lack of direct evidence as well as the scarcity and poor study of indirect evidence renders this topic highly speculative (Zherikhin 2002). The latter author concluded that the Palaeozoic record is not informative about detritivorous habits among roachoids (stem group of modern Dictyoptera, including Blattodea), and the disparate and diverse Mesozoic groups were most likely to have been opportunistic detritivores more specialized in habitat than diet, but direct evidence or clear indirect evidence of detritivory is lacking. Slater *et al.* (2012) showed several middle Permian cases of terrestrial detritivory through the study of coprolites, some of which they tentatively attributed to myriapods or insects.

There is no evidence of detritivory in Palaeozoic aquatic insects. Even if, at least by the late Permian, insects had already colonized aquatic environments, the diversity of aquatic insects was clearly lower during the Palaeozoic than during the Triassic (Sinitshenkova 2002, 2003). One reason could be that the Palaeozoic fossil record is rich in terrestrial insect adults but very poor in preimaginal stages, either terrestrial or potentially aquatic. Shcherbakov (2008) noted that ‘there are no reasons to assume a terrestrial mode of life in the Palaeozoic for preimaginal stages of those insect groups that now develop in the water’, but this reasoning ignores that substantial ecological differences could have existed between stem and crown groups of a clade. Kukulová-Peck (2009) studied a Carboniferous aquatic nymph of Meganisoptera (carnivorous Odonoptera). No further Permian nymph of this superorder is currently known, although all of the numerous and diverse ‘Protozygoptera’, Protanisoptera and stem Panodonata certainly had aquatic or subaquatic preimaginal stages. Few Triassic nymphs with a ‘zygopterous’ morphology are known in the Middle Triassic (Nel *et al.* 2022). Numerous mayfly nymphs are recorded from the transitional Permian–Triassic deposits of the Tunguska Basin, Siberia (Sinitshenkova 2013), but there are few Permian nymphs attributed to the stem group of Ephemeroptera, viz. *Kukulova americana* Demoulin, 1970 and *Misthodotes sharovi* Tshernova, 1965 (Demoulin 1970; Wootton 1988; Hubbard & Kukulová-Peck 1980; Sroka *et al.* 2021). Nearly nothing is known about the biology of these nymphs but they are supposed to have been herbivorous. Prokop *et al.* (2019) proposed that the nymphs of the Palaeozoic Paleodictyoptera had either aquatic or

amphibious ways of life. But their specialized sucking mouthparts were unlikely to have been adapted for detritivory. The archaeognathan family Dasyleptidae, potentially detritivorous as their extant relatives, could have been aquatic during the Carboniferous, Permian and Triassic, although this is strongly speculative (Sinitshenkova 2002, 2003). Bechly & Stockar (2011, p. 32) suggested 'a way of life at the marine and brackish drift line and possibly also freshwater shores' for these insects. Ponomarenko & Prokin (2015) considered that the Permian record of aquatic beetles is very scarce, much less abundant than the Triassic one. Furthermore, Prokin *et al.* (2019) described *Kargalarva permiosialis* Prokin *et al.*, 2019 from the upper Permian and attributed it to the beetle infraorder Schizophoromorpha. These beetles were likely to have been carnivorous. In contrast, the order Diptera, with numerous taxa having aquatic larvae, is hitherto unknown in the Permian but becomes very diverse in the Triassic, with numerous larvae, some of which could have been detritivorous.

CONCLUSION

The feeding habits determined based on detailed analysis of the exceptionally preserved cololites inside the most abundant mayfly nymph form in the lower Middle Triassic (Anisian) of Pedra Alta shed light on the early ecological role of insects in aquatic ecosystems. These mayfly nymphs were detritivores, recycling finely particulate organic matter in the early Mesozoic freshwater trophic net. This record represents the oldest direct evidence of detritivory known in insects. Furthermore, the sum of available evidence indicates that the detritivorous nymph of Pedra Alta was most likely to have been sedimentivorous, ingesting portions of the benthic muddy sediment. This lifestyle is extremely uncommon in insects and had not been hitherto reported in the palaeontological record. Sedimentivory apparently never became common in insects throughout their evolutionary history, including for the mayflies. Although the Pedra Alta mayfly nymphs could have been analogues of some extant burrowing species of the genus *Hexagenia*, an excavating habit cannot be determined at present for them. A detailed study of the mouthparts of this form will be crucial to increase the knowledge on their more specific feeding habits, although that will require the finding of additional material with exceptionally well-preserved heads.

The 3D, *in situ* cololite type described in detail herein is very scarce in the fossil record. The present work shows that fossilized gut contents, particularly if present *in situ*, are able to provide key palaeobiological data on palaeoenvironment and/or palaeoecological relationships.

It is unclear whether the end-Permian mass extinction event played a significant role in the constitution of early Triassic aquatic terrestrial ecosystems or whether it entailed an impoverishment of those existing beforehand, and how it affected the mode and tempo of the changes that took place. This uncertainty is caused by the extensive gap that exists in the fossil record of insect aquatic forms before the early Anisian. Thus, our knowledge on how the end-Permian mass extinction event influenced the different levels of the trophic guilds must await the discovery of older outcrops, particularly those of insects preserving their gut contents (Zherikhin 2002). For now, the rich outcrops of Grès à Voltzia (Gall & Grauvogel-Stamm 2005) and now Pedra Alta illuminate some key aspects of the early specializations of insects in aquatic communities, following the end-Permian mass extinction event.

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SUPPORTING INFORMATION

Additional Supporting Information can be found online (<https://doi.org/10.1002/spp2.1478>):

Figure S1. Preparation methodology of small sections of cololites on microscope slides using instant glue.

Figure S2. SEM images of the sample surfaces of a cololite (from section S1) from a specimen present in slab DA21/03-02-20 (specimen drawn in Fig. 6C), and the rock matrix near that cololite (Sa1 matrix), the elemental components of which were analysed (Figs 6, 8).

Figure S3. Camera lucida drawing of the components in a long portion of cololite from specimen 11 in DA21/14-02-01 (section S4, Figs 2, 7).

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