

Microscopy and egg morphology of Mayflies

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The chorionic structures of mayfly eggs can be categorized into three main classes (micropyles, attachment structures and chorionic sculpturing), according to their physiological function. Each class of structure shows a great variability as regards morphology, arrangement and distribution on the eggshell, and the combination of these features is the result of very particular chorionic patterns. The study of chorionic variability in both patterns and structures is very interesting for systematic purposes, because these features may be specific at different taxonomic levels. In addition, eggshell morphology allows us to identify female imagoes at species level when they lack valid taxonomic features, since eggs are completely formed in mature female nymphs. Light microscopy has already showed the great variability of the chorion structures in mayfly eggs and even allowed their classification. At present, this classification continues to be used as the basic reference in the morphological description of mayfly eggs, although it can only be done with SEM. Our morphological study on mayfly eggs, with both microscopy techniques, has allowed us to appreciate the beauty and complexity of the eggshell in this group of insects, as well as to describe new structures and to re-describe several aspects of chorion structures already known.

Keywords Micropyles; Attachment structures; Chorionic sculpturing; Ootaxonomy; Ephemeroptera; Egg morphology.

1. Introduction

Mayfly eggs are formed within the ovarioles, which are the individual unit constituting insect ovaries. Inside an ovariole, the differentiated oocyte follows a maturation process before it becomes an egg, by means of which it progressively stores reserve substances (yolk mainly), and is surrounded by a protective capsule, the eggshell [1]. The role of follicular cells in this maturation process is essential, because they promote and facilitate the entrance of yolk precursors to the oocyte and they are the only cells involved in eggshell synthesis. The mayfly eggshell is constituted by the viteline membrane and the chorion, and this last structure can also be formed of several layers [2-6], three of which can be distinguished at most. Some authors have noted an outer layer to the chorion, the extrachorion, although what its function is and where it is synthesized, within the ovariole or oviduct, have not been clearly defined [1, 7-10]. The particular biology of mayflies, whose imagoes usually have a very short life (days or hours), means that the oocyte maturation process takes place during the nymphal stage, so that the eggs are completely formed before the nymph emerges [7, 11]. Therefore, we can find eggs for morphological study in both mature female nymphs (with black wingpads) as in female imagoes.

The morphological study of eggshells may focus on their radial complexity, the ultrastructural relationships of the layers that constitute them, or their regional complexity, the distribution, organization and ultrastructure of the specialized areas of chorion surface [12]. Although knowledge of the radial complexity of eggshells in mayflies may be interesting from a biodiversity conservation point of view, this aspect has been studied less than the regional complexity, probably because data concerning the regional complexity has had an ample systematics application from a long time [8, 10, 13-17]. The chorionic features related to regional complexity may be specific at different taxonomic level in

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mayflies, and these have been used both to distinguish species and to establish phylogenetic relationships, although in this last case has not always been significant. In addition, the taxonomic usefulness of chorion features, together with the fact that the eggs are already formed in the nymphs before emerging, means that they can be used to identify the female imagoes, since these usually lack morphological features of taxonomic validity. We found evidence of increasing interest on systematics of egg morphology both in the articles that describe new species, since the incorporation of morphological data of the chorion together with those of nymphs and imagoes is common practice [18-24], and in the numerous studies that review egg morphology at any taxonomic level [6, 10, 14, 15, 25-30].

The size of mayfly eggs means that microscopy is necessary for their morphological study, since egg dimensions in most species are 150-260 μm long and 90-150 μm wide, although in few species the eggs may reach 1 mm in length [7, 11, 31]. Until the last third of the XX century, light microscopy showed that mayfly eggs have a great variability of chorionic structures, while Degrange [7] and Koss and Edmunds [32] established the conceptual bases for the morphologic descriptions of egg regional complexity in mayflies. These ample and deep studies continue to be considered a standard reference in the egg morphological descriptions of this insect group, even in those that are supported by scanning electron microscopy (SEM). According to these authors, chorionic structures can be grouped into three basic types (mycropiles, attachment structures, and chorionic sculpturing) each of which can also to be classified according to some characteristics of the chorion structures, such as like ultrastructure, single or collective arrangement, and position or distribution on eggshell surface. SEM studies of mayfly eggs confirm the validity of these classifications of chorionic structures, especially those proposal by Koss and Edmunds [32], and also allowing us to describe news types of structure and question the allocation of certain types of structures in eggs of some species described only with light microscopy [6, 10, 33]. However, SEM must not to be considered as the only microscopy technique for studying the eggshell morphology, since it has shown its inefficiency for observing structures that are partially within the chorion or which are masked by the extrachorion; in these cases, optical microscopy is more efficient although it does not provide the same degree of detail as SEM [6, 8-10, 33].

We have tried to characterize the regional complexity of mayfly eggshells at different taxonomic levels, based on a detailed analysis of the egg morphology of 29 species representing 18 genera and 8 families using SEM and light microscopy. We also attempt an overhaul of the relevant bibliography. Since the egg morphology of most analyzed species were either not known or had only been described by light microscope, we have had been able to describe new chorionic structures as well to re-describe different aspects from others. With this chapter, we would like to bring together the most relevant results of our study, as well make an approach to the regional complexity and beauty of the mayfly eggshell.

2. Materials and Methods

The mayfly species, whose eggs have been studied, were collected in the Segura river basin [34]. The eggs were obtained from mature female nymphs (with black wingpads) fixed in 4% formaldehyde and preserved in 70% ethanol, and from female imagoes fixed and conserved in 70% ethanol. In both cases, the eggs were extracted directly from specimens by abdominal dissection, introduced in nyal bags of 50- μm mesh, and kept in Eppendorf tubes with 70% ethanol until the beginning of the preparation procedure for both microscopy techniques used. Any manipulation of the eggs during the different stages of the microscopy preparation procedures was made in the nyal bags. Before the microscopy procedures, the eggs were cleaned in a Branson 3510 ultrasound bath for 5 min.

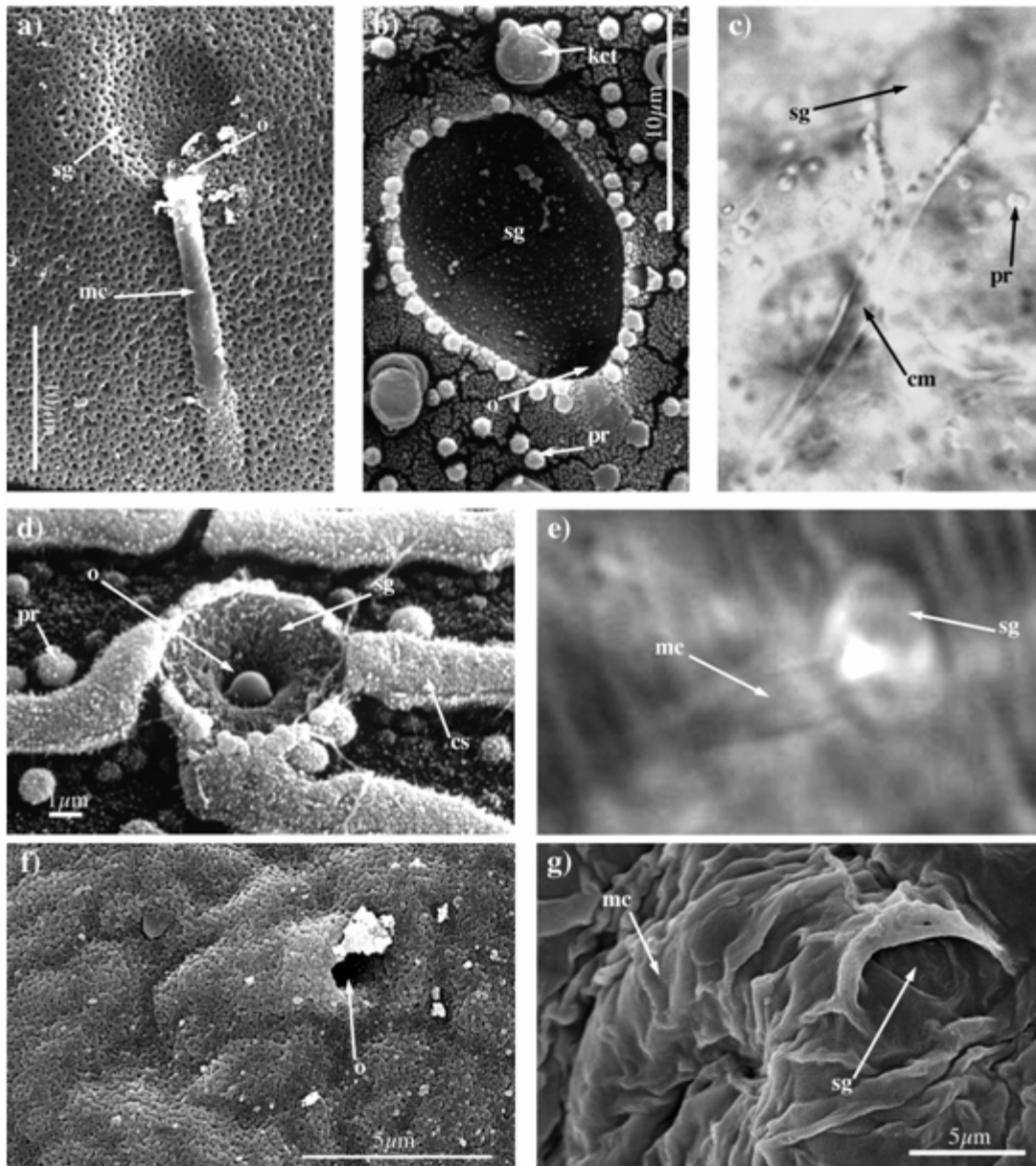


Fig. 1 Micropyles. a) Tagenoform type with micropylar canal running partially on surface in *Caenis pusilla*. b) and c) Tagenoform type in *Ecdyonurus dispar* with micropylar canal completely within chorion. d) and e) Funnelform type in *Habrophlebia* sp. f) Linear type in *Alainites* sp. g) Curved type in *Baetis lutheri*. Scale: 100x c) and b)

For light microscopy analysis, the eggs were processed in order to obtain the cleared and stained effect produced by the CMC-S mounting-medium used by Koss [11]. As this mounting-medium is no longer commercially available, the eggs were stained with neutral red and mounted on slides with “hoyer” mounting-medium. Three or four weeks after preparation, Eggs on slides were analyzed by a Leica DMRBB microscope.

For the SEM study, eggs were processed by the following procedure: dehydrated in increasing concentrations of ethanol (80%, 90%, 95%) until absolute ethanol or acetone, depending on the drying technique used: critical point or air-drying after hexamethyldisilazane/tetramethylsilane treatment [see 9]. They were stb mounted, sputter coated with gold-palladium and finally examined with Jeol JSM 6100 and Hitachi S3500N scanning electron microscopes. Forty to fifty eggs of each specimen studied were prepared for SEM analysis, verifying the morphological characteristics of the chorion in at least 10 of them.

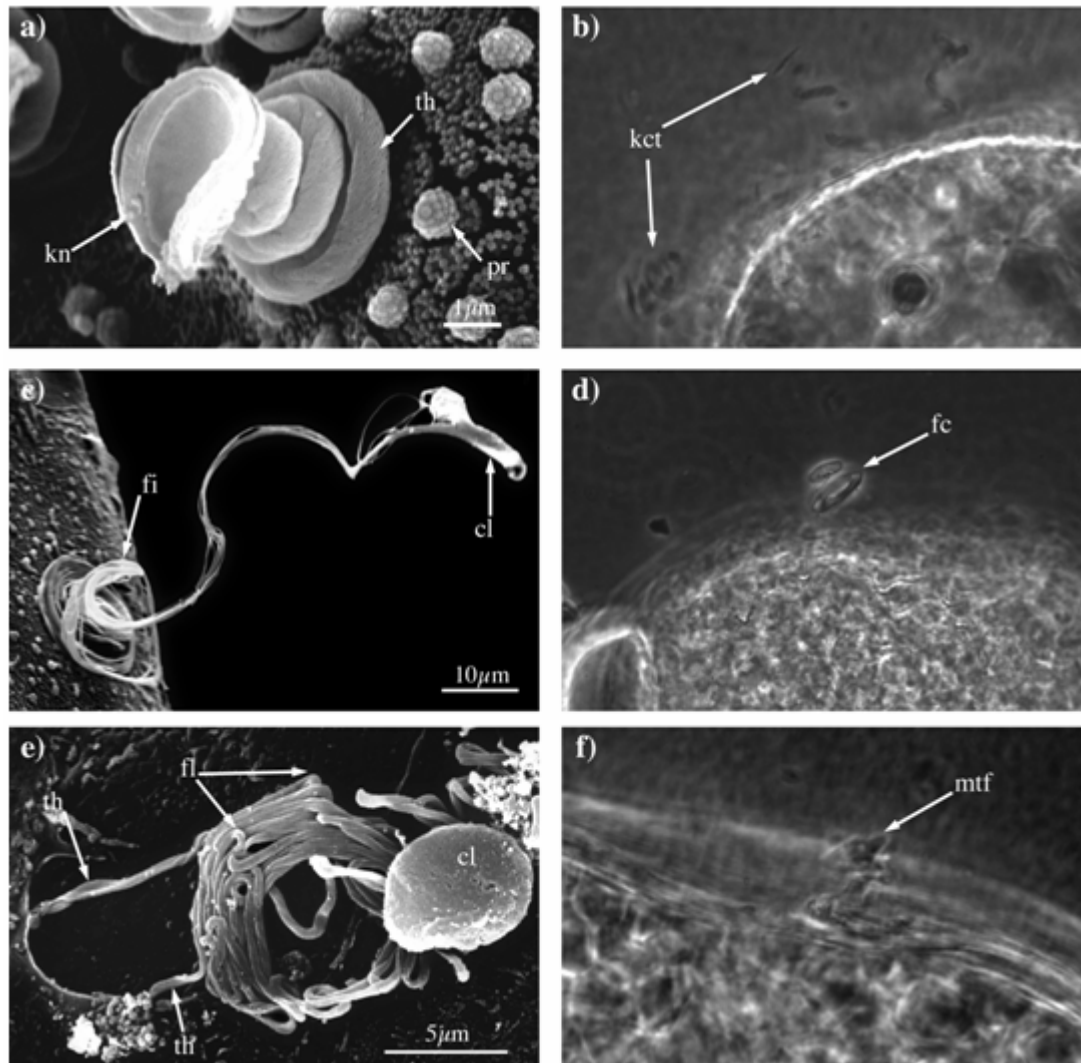


Fig. 2 Morphology comparison of lateral attachment structures by SEM and light microscopy. a) and b) Knob terminated coiled thread (KCT) in *Ecdyonurus dispar*. c) and d) Fiber-coil with terminal fiber clusters in *Potamanthus luteus*. e) and f) Multi-threads folded with terminal fiber clusters (MTF) in *Serratella ignita*. Scale: 100x c), d) and f)

Abbreviations used in figures: al-ex = complex adhesive-extrachorion layer; cl = fiber cluster; cs = costa; ev = viteline membrane; fb = fiber layer; fc = fiber coil with terminal fiber cluster; fi = fiber; fl = folded; kct = knob-terminated coiled thread; kn = knob; mc = micropylar canal; mp = micropyle; mrt = microrreticulum; mtf = multi-thread folded with terminal fiber cluster; o = micropylar opening; or = orifice; pc = polar cap; pr = protuberance; rt = reticulum; sg = sperm guide; tb = chorion internal trabecular; th = thread.

3. Results and Discussion

The terminology and classification of the chorion structures proposed by Koss and Edmunds [32] have been followed in this study. Basically, chorion structures can be categorized into three main classes according to their physiological function, a) micropyles, structures allowing the sperm to enter the oocyte, b) attachment structures, allowing egg fixation on substrate in order to avoid egg-drift, and c) chorionic sculpturing, structures still without a well-known function. Some authors have proposed that mayfly eggs present another basic chorion structure, aeropyles, which are related to gaseous interchange during the embryonic development [2, 6, 9, 10]. In the following sections, we will offer an overview of the great variability of these basic types, adding our contributions to the knowledge of eggshell morphology.

3.1 Micropyles

Three parts can be distinguished in the mayfly micropyle: the sperm guide, the micropylar opening and the micropylar canal. The sperm guide is a conspicuous depression on the chorion whose form usually varies from round to oval, although in some cases it can be poorly defined or even lacking (Figure 1). The micropylar opening is a simple orifice that begins the micropylar canal, and whose shape reflects the initial section of this tube. Since the micropylar canal is a tube that crosses the chorion, it cannot generally be observed by SEM, as that the sperm guide and the micropylar opening are the only observable parts of the micropyle using this technique (Figure 1), except in *Caenis* eggs [6, 30], where the micropylar canal runs partially on the chorion surface (figure 1a). The shape and length of micropylar canal can only be studied by light microscopy (Figure 1c,e). The extrachorion does not usually cover the external parts of the micropyle, although in some species of *Caenis* it may cover the sperm guide but never the micropylar opening [6].

Several aspects of micropyle organization, such as the definition of the sperm guide or the position of both micropylar opening and canal with respect to the sperm guide, allow different types to be distinguished. Until now, three types of micropyles had been established, *tageniform*, *funneliform* and *linear* (Figure 1a-f) [7, 11, 32]; we now think that a new type of micropyle can be defined in Baetidae eggs, the *curved* one (Figure 1g), based on the curved trajectory of the micropylar canal when introducing itself into chorion. Finally, the number of micropyles that can appear in mayfly eggs is very variable, ranging from very numerous (< 3 units) as in the cases of *Serratella*, *Ecdyonurus* and *Rhitrogena* (Figure 5d) [6, 17], to one to three units, as is the case of *Caenis*, *Baetis*, *Habrophlebia* and *Habroleptoides* (Figure 5a-f, h) [6, 27-30].

3.2 Attachment Structures

The chorionic structures that present the greatest morphologic variability are the attachment structures, of which Koss and Edmunds [32] made an exhaustive classification from data concerning their ultrastructure, organization when they are packing, and distribution on the eggshell. According to this classification, the attachment structures may be of a fibrous (Figure 2) or non-fibrous nature (Figure 3a,d); they may be distributed uniformly on the whole egg as a layer (Figure 3 a,d) or to be located discreetly in a specific place of the egg, being denominated polar cap if they appear in the polar zones of egg (Figure 5b,d,g) or lateral attachment structures if they appear in any other egg part (Figure 5d); they may be formed of only one filament (monofilament ones) or numerous filaments (polyfilament ones) (Figure 2), which may be loosely arranged (fibers) or tightly torsioned unit (thread); they may be a coiled-packed or non-coiled packed (Figure 2); etc. Indeed, the possible combinations of these parameters that we can find among the attachment structures are multiple.

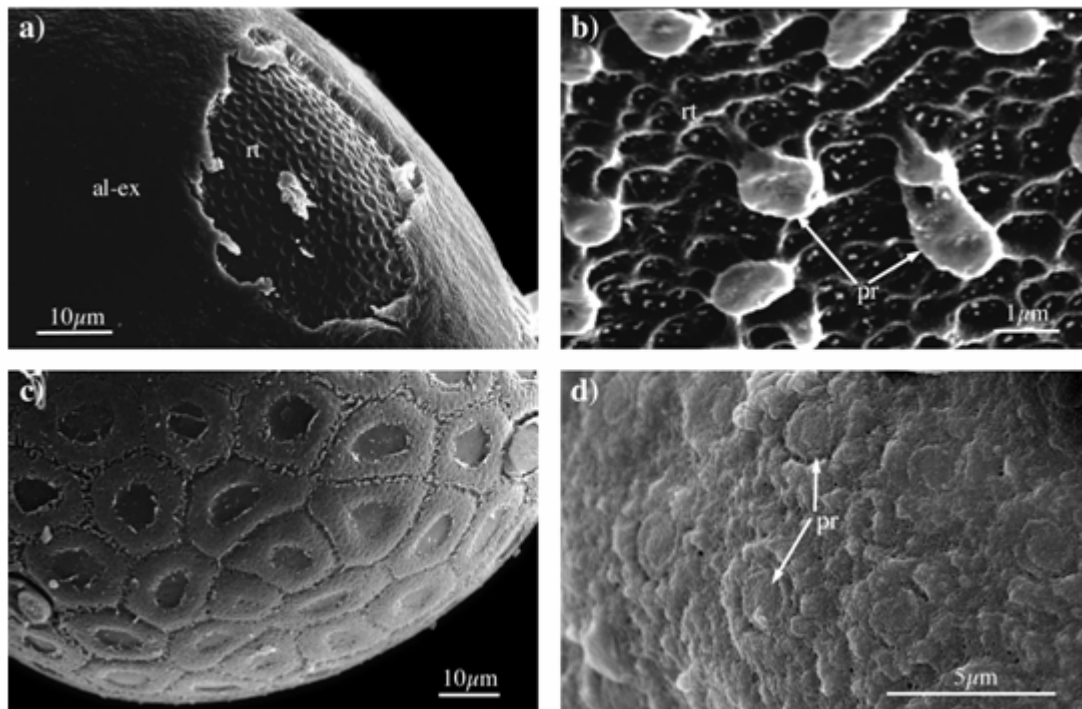


Fig. 3 Chorionic sculptuting. a) small-mesh regular reticule and extrachorion-adhesive layer complex in *Ephemerella danica*. b) Small-mesh irregular reticule and tuberculate-like protuberances in *P. luteus*. c) Wide-mesh regular reticule in *Serratella spinosa*. d) Protuberances complex in *Cloeon inscriptum*.

We were unable to check all types of attachment structures defined in the classification mentioned above, because they did not always appear in the studied eggs. But, we have been able to re-describe several types as knob terminated coiled thread (KCT), fiber-coils with terminal fiber cluster, fibrous and non-fibrous layers, three types of polar cap (I, III, V), as well as other attachment structures not previously described. KCT is the commonest attachment structure among mayfly species, and has been described in eggs of Heptageniidae, Potamanthidae and Ephemerellidae [8, 21, 26, 27, 32, 33]; however, our data shows that KCT only appear in eggs of Heptageniidae species studied. Probably, the similar shape that these structures show by optical microscope was the cause of this confusion (Figure 2b,d,f), because the ultrastructure and organization of each one are clearly different when observed by SEM (Figure 2a,c,e). Fiber-coil with terminal fiber clusters is the attachment structure in Potamanthidae, since the polyfilament is constituted by a loose collection of filaments (Figure 2c) [10]. Multi-threads folded with terminal fiber clusters (MTF) is the new type of lateral attachment structure that we have observed in Ephemerellidae, where it is characterized by two thread weakly twisted which are folded in zig-zag to pack themselves (Figure 2e) [6].

Extrachorion and attachment structures are closely related, because the extrachorion usually covers the attachment structures, especially those that are fibrous, probably in order to avoid accidental unpacking, although this has not been demonstrated experimentally. On the other hand, the extrachorion can prevent the correct observation of the attachment structure, and so must be considered in the morphological studies of chorion.

3.3 Chorionic sculpturing

Mayfly eggshells show different chorionic structures whose functions in the egg are still not known but, although they are very diverse, they can be grouped in the same basic type called chorionic sculpturing. In most of cases, the shape of chorionic sculpturing is the reflection of the morphology of the follicular cells, which is why they are also denominated follicular cells impressions, and usually constitute reticular structures (Figures 3a-c). The reticules can form regular or irregular geometric reliefs (Figures 5c,e) and, according to the size of its mesh units, they can be considered to be of large mesh (Figure 3c) or small mesh, as we describe for first time in *Potamanthus* and *Ephemera* (Figure 3a,b). The microreticules, a new type of chorionic structure that we have described in eggs of several Baetidae genera (*Baetis*, *Acentrella* and *Alainites*) (Figure 4b), are characterized by their very small mesh size (< 0.5 μm of wide). But, we also found different types of not reticular chorionic sculpturing, which are very variable in both shape and size; for example protuberances (tubercle-like, crest-like, etc) (Figure 3b,d), ribs (Figures 4d and 5a,f), pores (Figure 1a), depressions, etc.

The extrachorion can mask the chorionic sculpturing, and so must be considered in morphological studies of eggshell. In some cases, as in *Ephemera danica*, the combination of attachment structure and extrachorion, which form a thick layer surrounding the chorion, prevent the SEM observation of chorionic sculpturing (Figure 3a), although it is observable by light microscopy.

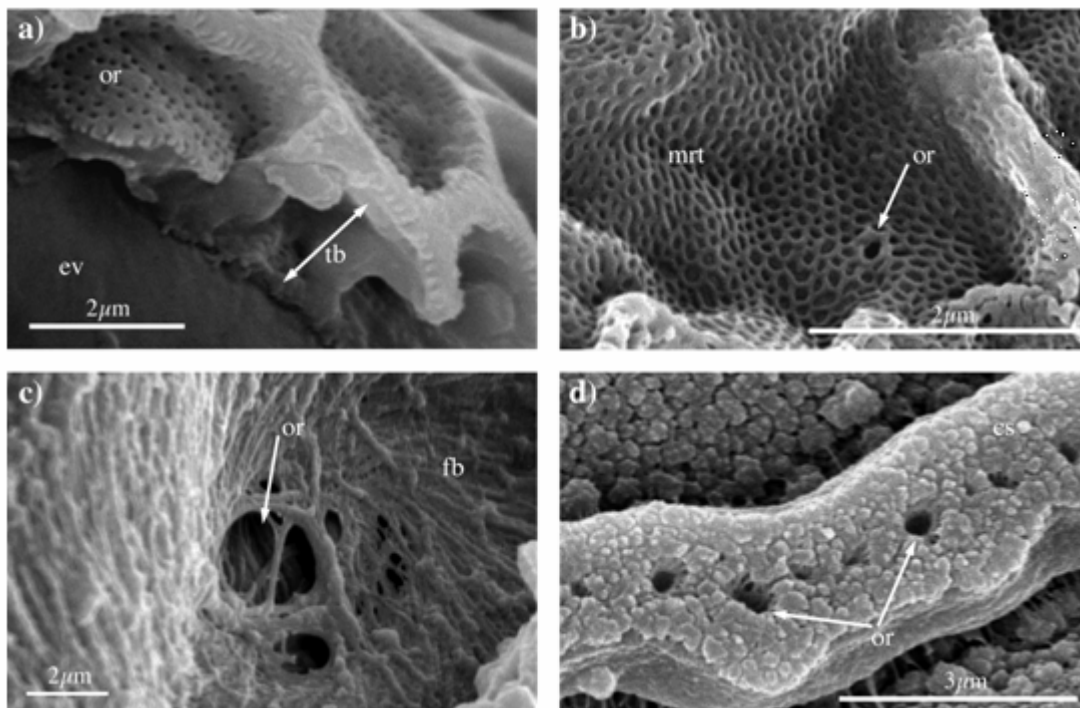


Fig. 4 Aeropyles. a) Trabecular aeropyle in *Centropilum luteolum*. b) Microrreticule and aeropylar orifice in *Baetis maurus*. c) Fiber layer and aeropylar orifice in *Oligoneuriella rhenana*. d) Costa or rib with aeropylar orifices in *Habrophlebia fusca*.

3.4 Aeropyles

Hinton [35] considers aeropyles those orifices of the chorion that measure 1-0.2 μm and that can communicate with the interior of the egg either directly or through an internal trabecular zone of chorion. Although this type of structure has only been described once [2], we have found chorionic orifices in

different species that could have an aeropylar function [6, 9], although a TEM study is necessary to confirm this. We found the most evident aeropylar structure in *Centroptilum luteolum*, where the numerous orifices of each mesh unit open up into an internal trabecular cavity of the chorion (Figure 4a). The rest of the aeropylar structures that we thought to have detected correspond to orifices of different sizes in eggs from *Oligoneuriella rhenana*, *Habrophlebia fusca*, and several species of Baetidae (Figure 4 b-c).

3.5 Egg chorionic patterns

The diverse combinations of the basic types of chorionic structures that we have described previously were the result of different chorionic patterns (Figure 5) which, in some cases, can be used to characterize the egg at genus level (*Acentrella*, *Caenis* or *Choroterpes*) (Figure 5a,c,f), or family level (Potamanthidae) (Figure 5d) [6, 10, 18, 20, 23, 30]. In these taxa, the egg morphology is only well-known in a few species and probably, as in other genera and families, this taxonomic characterization is not maintained when the egg is described in a greater number of species. In any case, each supraspecific taxa normally has a chorionic pattern that it is considered most common, since it is shared by the greater number of species. At genus level, the most common chorionic patterns in *Habrophlebia* show longitudinal costae or ribs running from one pole to another (Figure 5f) [1, 2, 6] and, at family level, the commonest chorionic patterns in Heptageniidae show numerous lateral attachment structures of KCT type (Figure 5g) [6, 10, 14, 16, 26]. In this last case, the chorionic patterns of *Epeorus* differ enormously, because it lacks any type of lateral attachment structure (Figure 5h) [6]. The extrachorion can also mask the chorionic pattern, as has been described in *Potamanthus luteus* [9, 10].

4. Conclusion or General Remarks

For its correct study, the regional complexity of the mayfly egg requires the joint use of light microscopy and SEM, since both techniques are complementary and necessary for a global knowledge of eggshell morphology [10, 33]. But, since SEM is technique most commonly used at present for this kind of study, a redescription of egg morphology is necessary in those species which, to date, have only been studied by light microscopy. In addition, as we have shown, new types of structure exist and remain to be described, while some types of chorionic structure need to be re-assigned in numerous eggs.

The extrachorion is a thin film which completely surrounds the egg, and has been detected in most studied species [9]. Unlike in other insect eggs, the functions of extrachorion in mayfly eggs are still unclear. This layer can represent a serious problem when studying egg morphology by SEM, since it may mask chorionic structures. It is therefore necessary to know how to recognize it in order to avoid the erroneous interpretation of the chorionic pattern or arrangement of some chorionic structures. At the moment we are working on a procedure for eliminating this structure from the egg.

The systematic usefulness of eggshell morphology in mayflies has been widely demonstrated [13, 14, 30, 32], although in some families or genera it has not helped to solve existing phylogenetic problems [8, 17]. From a taxonomic point of view, as we mention [6], eggshell morphology has allowed the unequivocal specific identification of specimens studied, although it is necessary to continue studying more species in order to confirm this.

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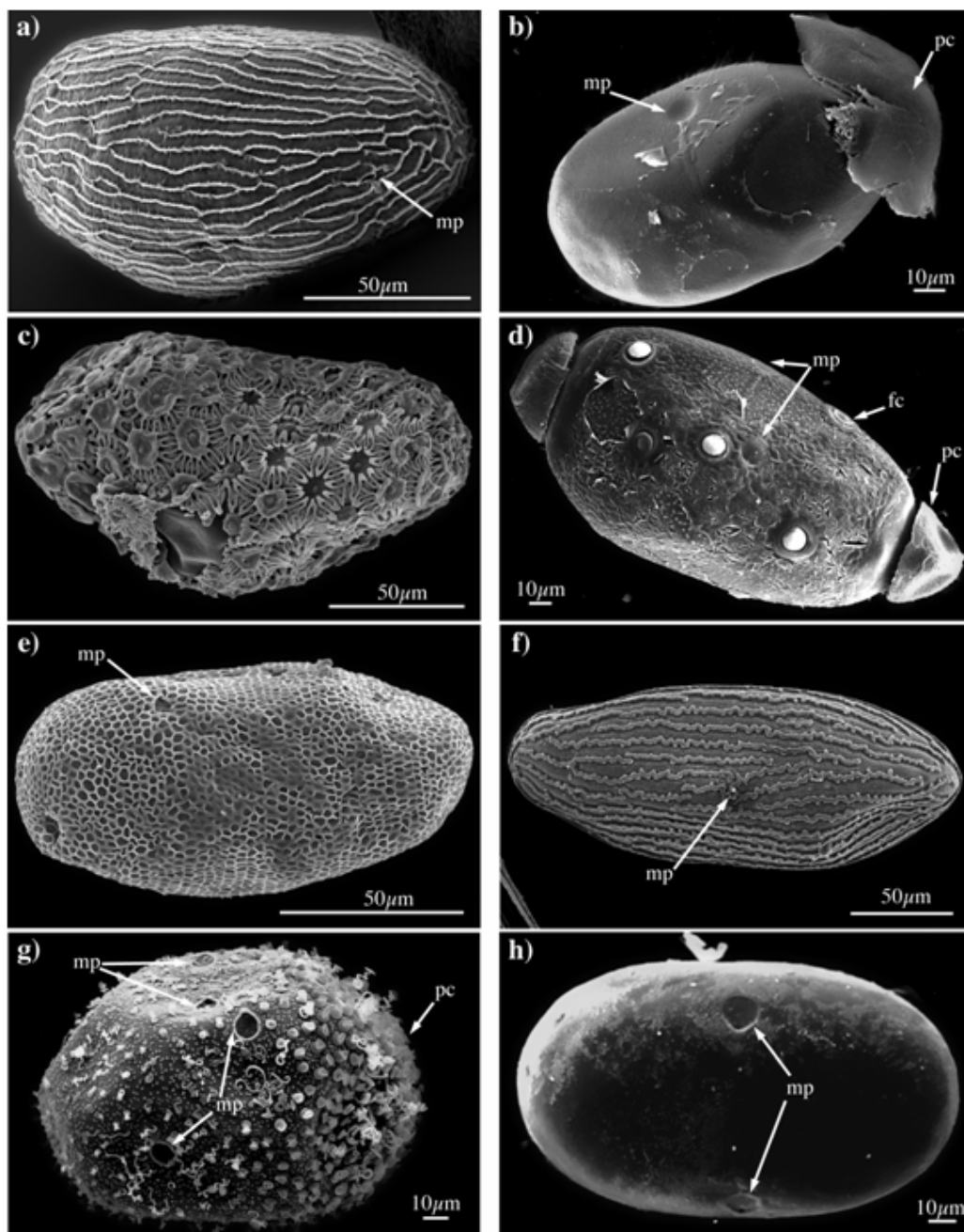


Fig. 5 Chorionic patterns. a) *Acentrella almohades*. b) *Caenis pusilla*. c) *Choroterpes prati*. d) *Potamanthus luteus*. e) *Centroptilum luteolum*. f) *Habrophlebia fusca*. g) *Ecdyonurus aurantiacus*. h) *Epeorus torrentium*.

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