

New type of egg attachment structure in Ephemeroptera and comparative analysis of chorion structure morphology in three species of Ephemerellidae

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Abstract

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Ephemeroptera eggs vary greatly in their morphology, a characteristic that has been used to solve systematic problems. The eggs of Ephemerellidae may be considered among the most studied in mayfly families, especially with regard to the number of species for which they have been described (approximately 32 species corresponding to 12 genera). However, this study provides new details of the egg morphology of three species of this family, *Serratella ignita* (Poda 1761), *S. spinosa* (Ikononov 1961) and *Torleya major* (Klapalek 1905). The main finding was that the morphology and organization of the lateral attachment structures do not resemble the type normally associated with ‘*knob terminated coiled thread*’ (KCT). The particular characteristics of this structure, never before described for Ephemeroptera, led us to propose a new type of attachment structure, the ‘*multithread-folded with terminal fibre cluster*’ (MFT). We also provide a detailed description of the chorion morphology of the eggs of the three species, identifying a tear-shaped variation in the oval sperm guide and confirming the type III polar caps in the two species of *Serratella* Walsh, 1862.

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Introduction

Regional complexity was proposed by Margaritis (1985), to describe, basically, the position and arrangement of functional chorionic structures in eggs of the dipteran *Drosophila melanogaster* Meigen, 1830. This complexity in Ephemeroptera eggs is related to three basic types of chorionic structure, already defined by Koss and Edmunds (1974): the attachment structures, the micropyle and chorionic sculpturing. The characteristics of a structure, or a combination thereof, may be particular to different taxonomic levels, so that they have frequently been used to establish phylogenetic relationships (McCafferty and Wang 1994; Studemann and Landolt 1997; Domínguez and Cuezco 2002). Moreover, given that the egg is completely mature at the end of the nymph stage, the characteristics of the chorion allow us to associate

nymphs and female imagos, which is very useful since the latter usually lack taxonomically valid morphological characters (Koss 1968). The chorion morphology of Ephemeroptera eggs has been studied in numerous families (Degrange 1960; Koss and Edmunds 1974; Kopelke and Müller-Liebenau 1981a,b, 1982; Malzacher 1982; Gaino *et al.* 1987, 1993; Kluge *et al.* 1995; Klonowska-Olejnik 1997; Klonowska-Olejnik & Jazdzewska 2003; Ubero-Pascal 2004; Ubero-Pascal and Puig 2007), although the number of species in which they have been studied in depth is still low.

Attachment structures are essential for egg viability after ovoposition, since they are responsible for fixing eggs firmly to the substrate and so prevent them from drifting (Gaino and Reborá 2003); they are also important from a morphological point of view since it is the chorionic structures that show greatest variability. This variability is evident in their

ultrastructure, arrangement and position or distribution on the egg surface. Koss and Edmunds (1974) proposed a classification of Ephemeroptera attachment structures, although they also stated that this could change as the egg morphology of more species became known. Indeed, given their biological importance and morphological diversity, attachment structures have been the main focus in a large number of the studies on Ephemeroptera eggs (Gaino and Mazzini 1989; Gaino and Bongiovanni 1991, 1992; Gaino and Rebora 2001; Ubero-Pascal 2004; Ubero-Pascal and Puig 2007), the findings of which have provided more detail and shown the need to update Koss and Edmunds' classification.

Compared with other Ephemeroptera, such as Potamanthidae, Ephemeridae and Oligoneuriidae, the egg morphology of Ephemerellidae has been studied in a considerable number of species (Ubero-Pascal and Puig 2007). The characteristics of the chorion have been established both by optical microscopy and scanning electron microscopy (SEM) in approximately 32 species, belonging to 11 of the 22 genera forming this family according to Hubbard (1990); however, most of the descriptions given have been quite superficial (Bengtsson 1913; Smith 1935; Degrange 1956, 1960; Koss 1968; Koss and Edmunds 1974; Studemann and Tomka 1987; Gaino and Bongiovanni 1992; Kang and Yang 1995; Studemann *et al.* 1995; Studemann and Landolt 1997; Haybach 2003; Jacobus and Sartori 2004; Jacobus *et al.* 2004). In systematics, the application of the morphology of Ephemerellidae eggs has provided patchy results, although it does seem that egg characteristics are species-specific, for which reason they are of great taxonomic value at this level, but not so much at supra-species level or for clarifying their phylogeny (McCafferty and Wang 1994; Studemann *et al.* 1995; Studemann and Landolt 1997).

In this study, we analyse in detail the egg morphology of three species of Ephemerellidae, *Serratella ignita* (Poda, 1761), *S. spinosa* (Ikononov, 1961) and *Torleya major* (Klapalek, 1905), using optical microscopy and SEM. The results enabled us to confirm the most common chorionic pattern in the eggs of this family and the specific pattern of each species. However, we found differences in the ultrastructure and organization of the attachment structures, especially in the lateral attachment structures, which do not fit the pattern of 'knob terminated coiled thread' (KCT). Furthermore, the characteristics observed in the lateral attachment in these three species do not reflect any types established by Koss and Edmunds (1974), leading us to denominate a new type of structure. After studying the figures in several works published on Ephemerellidae eggs, we consider that this type of structure may appear in many other species and could be considered specific to this family.

Materials and methods

The three species whose eggs have been studied were collected in the Segura river basin, S. E. Spain (Ubero-Pascal

et al. 1998): three nymphs and one imago of *S. ignita* from the Segura river near La Graya village (615 m, 20 March 1994 and 13 Nov. 1994, respectively); three nymphs of *S. spinosa* from the Mundo river near Los Chorros (985 m, 10 July 1994); and two nymphs of *T. major* from the Madera stream near Segura de la Sierra village (1180 m, 10 July 1994). Female mature nymphs (with black wingpads) were fixed in 4% formaldehyde and conserved in 70% ethanol, whereas imagos were fixed and preserved in 70% ethanol. Eggs from the imago were studied only in the case of *S. ignita*.

The eggs of each species were obtained directly from the abdomen of specimens and were processed for SEM by cleaning in a Branson 3510 ultrasound bath for 5 min and dehydrating in increasing concentrations of ethanol (80%, 90%, 95%) until absolute ethanol or acetone. They were then dried using the critical point method or air-dried after hexamethyldisilane/tetramethylsilane treatment (see Ubero-Pascal *et al.* 2005), mounted on stubs with conductive sticky tape, and sputter coated with gold-palladium in a Polaron Sputter Coater. Finally, the eggs were observed by scanning electron microscopes (Jeol JSM 6100 and Hitachi S3500N) with working voltages of 20 and 10 kV, respectively. Forty to fifty eggs of each specimen were prepared for SEM analysis, checking the morphological characteristics of the chorion in at least 10 of them.

For light microscopy analysis, the eggs were processed in order to obtain the cleared and stained effect produced by CMC-S mounting-medium that Koss (1968) used. As this mounting-medium is no longer commercially available, the eggs were stained with neutral red and mounted on slides with 'hoyer' mounting-medium. Eggs on slides were analysed 3 or 4 weeks after preparation by a Leica DMRBB microscope.

The terminology and classification of the chorion structures proposed by Koss and Edmunds (1974) were followed in the descriptions of egg morphology. Koss (1968) used the mesh length to compare reticle size in different species, considering this parameter as the longest inside dimension of the mesh unit (excluding the strand). However, we consider this parameter to be of little use in geometric reticle composed of regular and irregular polygons, or only irregular, as occurs in some of the species studied. We therefore think that comparisons of mesh size should be based on three parameters: the mean length of the polygon sides, the mean distance between opposite vertices and the mean distance between opposite sides.

Results

Morphology and arrangement of lateral attachment structure

In Ephemerellidae the lateral attachment structures are fibrous and take on the circular shape of the depression when packed (Fig. 1A,B). These structures are mainly situated in the subpolar zones of the egg and vary in number (Fig. 4),

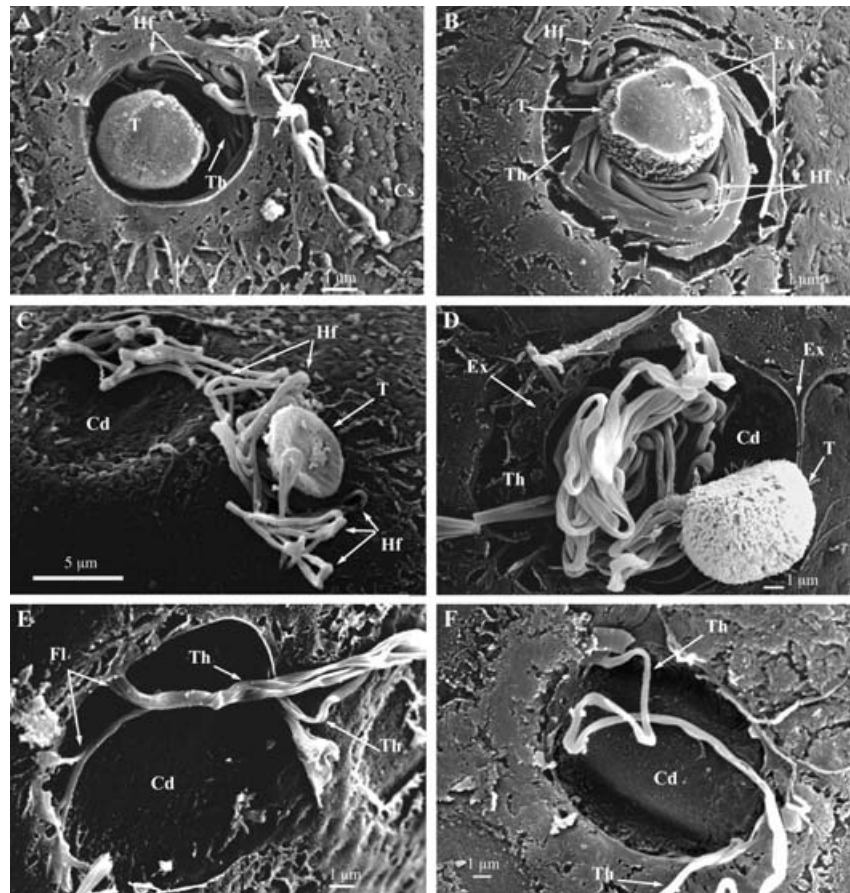


Fig. 1—Ultrastructure and organization of multithreads-folded with terminal fibre cluster (MFT). —**A, B**. Packing arrangement covers partially of extrachorion. —**C, D**. Unravelled configuration. —**E, F**. Detail of threads joined to the chorion at the edge of circular depression. *S. ignita* (A and C). *T. major* (B and F). *S. spinosa* (D and E). Cd, circular depression; Cs, chorion surface; Ex, extrachorion; Fl, filament; Hr, hairpin folds; La, lateral attachment structure; T, terminal fibre cluster; Th, thread.

although they may occasionally be shifted towards the equatorial area or the pole without a cap. The packed fibres in each unit are arranged in a slight depression in the chorion, although this depression is only visible when they are absent or unraveled (Fig. 1C,E,F); the whole unit is kept in place by a thin film known as the extrachorion (Figs 1A,B). When this extrachorion is broken or becomes detached, the fibrous material unravels and reveals its ultrastructure and organization (Figs 1E,D and 3A). Basically, each lateral attachment structure is made up of at least two threads and a circular cushion (Fig. 1C–F). The threads are joined to the chorion just at the edge of the depression at different insertion points (usually diametrically opposed to each other; Figs 1E,F). Each thread is formed of three or four flattish filaments, weakly twisted and attached to the edge of the depression in several places (Fig. 1E). The threads are joined distally to the same place on the centre of the lower side of the circular cushion. The cushion is formed of numerous microfilaments that give it a velvety appearance (Fig. 1B,D).

What is surprising about this structure is the way in which the packed threads are organized, since they do not show the spiral common to other attachment structures in Ephemeroptera. This organization is represented schematically in Fig. 2, where the zigzag folding of the threads within the depression

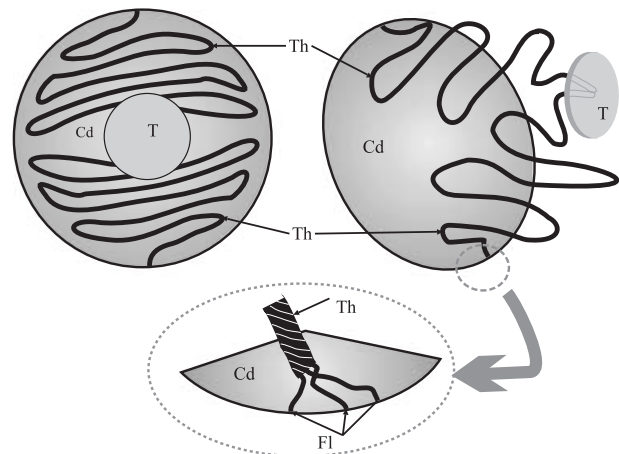


Fig. 2—Schematic representation of basic organization of MFT and detail of proximal end of thread. Cd, circular depression; Fl, filament; T, terminal fibre cluster; Th, thread.

is depicted. This folding completely covers the surface of the depression, in the centre of which the cushion can be seen (Fig. 1B). This particular arrangement of the threads is more evident when the structure has unfolded (Fig. 1E,D),

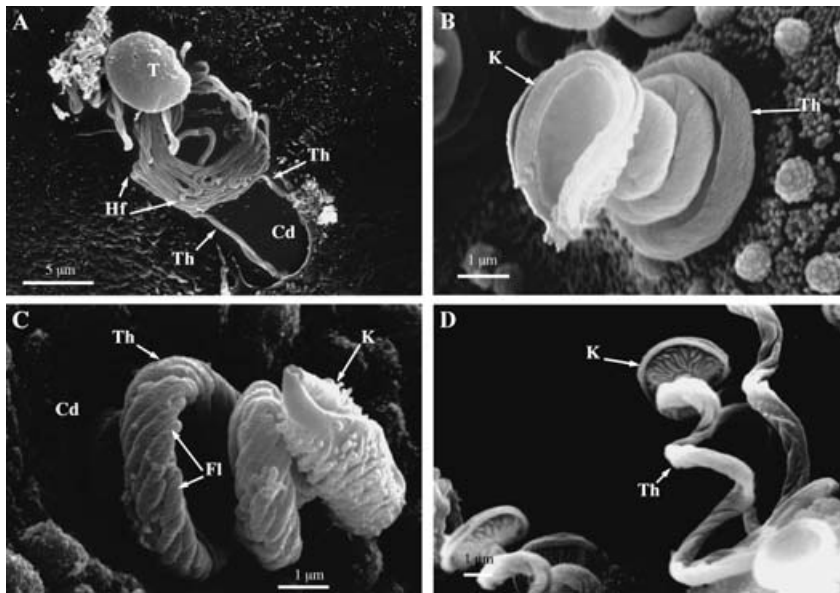


Fig. 3—Comparison of organization and structure of MFT and KCT. —**A.** *S. ignita*, MFT partially unravelled. —**B.** *Ecdyonurus dispar* (Curtis, 1834), packed KCT. —**C.** *Rithogenia iridina* (Kolenati, 1839), partially unravelled KCT with detail of twisted filaments of the thread. —**D.** *E. dispar*, uncoiled KCT showing the thread spiralled. Cd, circular depression; Fl, filament; Hf, hairpin folds; K, knob; T, terminal fibre cluster; Th, thread.

Table 1 Koss and Edmunds (1974) attachment structures with the new attachment structure described

I.- Non-fibrous	Sucker-like disc or plates Projections Layers			
II.- Fibrous	II.A.- Non-coiled	II.A.1.- Layers II.A.2.- Threads or fibres	Lateral Polar cap type I Polar cap type II	
	II.B. Folded II.C.- Coiled	Multi-thread with terminal fibre clusters II.B.1.- Fibres II.B.2.- Collectively coiled threads II.B.3.- Individually coiled threads	Without terminal fibre clusters With terminal fibre clusters With terminal knob Polar cap type III II.B.3.a.- Knobless II.B.3.b.- Knob-terminated	Lateral Polar cap type IV Lateral Polar cap type V

although it also is clear when the extrachorion permits it to be seen (Figs 1B and 3A). In this last case, since the cushion terminal is smaller than the complete structure, the hairpin folds that make up the thread when it is folded can be seen (Figs 1B and 3A).

Until now, these attachment structures have been regarded as being of the 'KCT' type. These KCTs are common in the eggs of Heptageniidae and are formed of one thread made up of tightly entwined filaments (Fig. 3C), which finish in a circular knob (Figs 3B,C). In this type of structure the thread is coiled (Fig. 3B), giving it the form of a spring when it expands (Fig. 3D). The same type of structure may also be associated with a circular depression, in which case the thread is joined to the centre of the depression (Fig. 3C). Clearly, these morphological characteristics differ substantially from

those described above in the Ephemerellidae species studied (Figs 1 and 3A). For this reason, we do not think they should share the same name and propose they should be designated 'multithread-folded with terminal fibre clusters' (MFT), and, as such, be included in Koss and Edmunds' classification (1974) as a new fibrous structure type, as depicted in Table 1.

The size of the MFT in the species studied cannot be considered uniform (Table 2), and is considerably smaller in *S. ignita* than in *S. spinosa* and *T. major*. The same may be said of the thread thickness (Table 2), although the diameter of the circular cushion is more homogeneous and almost identical in the case of *S. ignita* and *T. major* (Table 2). In addition, we have seen that in the eggs of *S. spinosa* the lateral attachment structures in the subpolar region near the cap are larger (14.6 µm mean diameter) than the structure near the

Table 2 Chorion structure measurements

	<i>Torleya major</i>	<i>Serratella ignita</i>	<i>Serratella spinosa</i>
Multithread-folded with terminal fibre cluster			
Number of structures*	4–5	11	8–9
Diameter of terminal fibre cluster	6.4	6.2	7.2
Diameter of Circular depression	13.2	9.8	13.6
Thread thickness	0.6	0.38	–
Micropyles			
Units number*	2–3	4–7	5
Sperm guide form	Circular	Oval/Teardrop†	Teardrop
Sperm guide size	14	Long 8.4/10.6†	Long 18.25
		Large 8.7/6.3†	Large 10.7
Netlike Chorionic sculpturing			
Side length	9.6	–	9.5
Length between sides	16.9	–	16.5
Length between apices	19.2	–	18.6

All average data given in μm . $n = 15$.

*Units observed directly in the eggs, nonextrapolable data of maximum number of units in the whole egg.

†Teardrop sperm guide measurements.

Table 3 Comparison of egg size in studied species

	<i>Torleya major</i>		<i>Serratella ignita</i>		<i>Serratella spinosa</i>	
	Length	Width	Length	Width	length	Width
Our data	168*	125	145*	109	189*	141
	178†		160†		210†	
Degrange (1960)	203–222*	144–156	175–211*	136–160	–	–
Studemann <i>et al.</i> (1995)	200‡	–	250‡	–	340‡	–
Gaino and Bongiovanni (1992)	–	–	–	132	–	–
	–		213†		–	–

All data are given in μm . All data indicated are mean data, except Degrange's data. $n = 15$.

*Egg length without measuring the polar cap length.

†Egg length including the polar cap length of bulged type according to Gaino and Bongiovanni (1992).

‡Average length of egg considering polar caps of different types and lengths.

opposite pole (11.21 μm mean diameter). Such a positional variation in the size of the lateral attachment structure was not observed in the other two species studied.

Egg morphology of studied species

The eggs of the three species studied (Fig. 4) share the same morphotype, characterized by their ovoid shape, one polar cap, numerous lateral attachment structures and numerous tageniform-type micropyles, although the chorionic sculpturing varies. The mean egg size of *S. ignita* is considerable smaller than in the other two species, *S. spinosa* having the largest eggs. In general, the mean egg size in our study was

considerably smaller than the values recorded in the literature (Table 3).

Optical microscope and SEM images of the polar caps of the eggs of the three species show them to be made up of innumerable filaments, bulbous at one end – spherical in *S. ignita* and *T. major* (Fig. 5A) and bell-shaped in *S. spinosa* (Fig. 5D). The polar cap is made up internally of a dense amorphous-looking matrix, which forms a central nucleus from which the filaments arise (Fig. 5E). The nucleus is joined to the chorion by means of a conical projection (Fig. 5G,H). The filaments are densely packed because of the extrachorion that covers them, although it can be seen that they are weakly wound around a central nucleus.

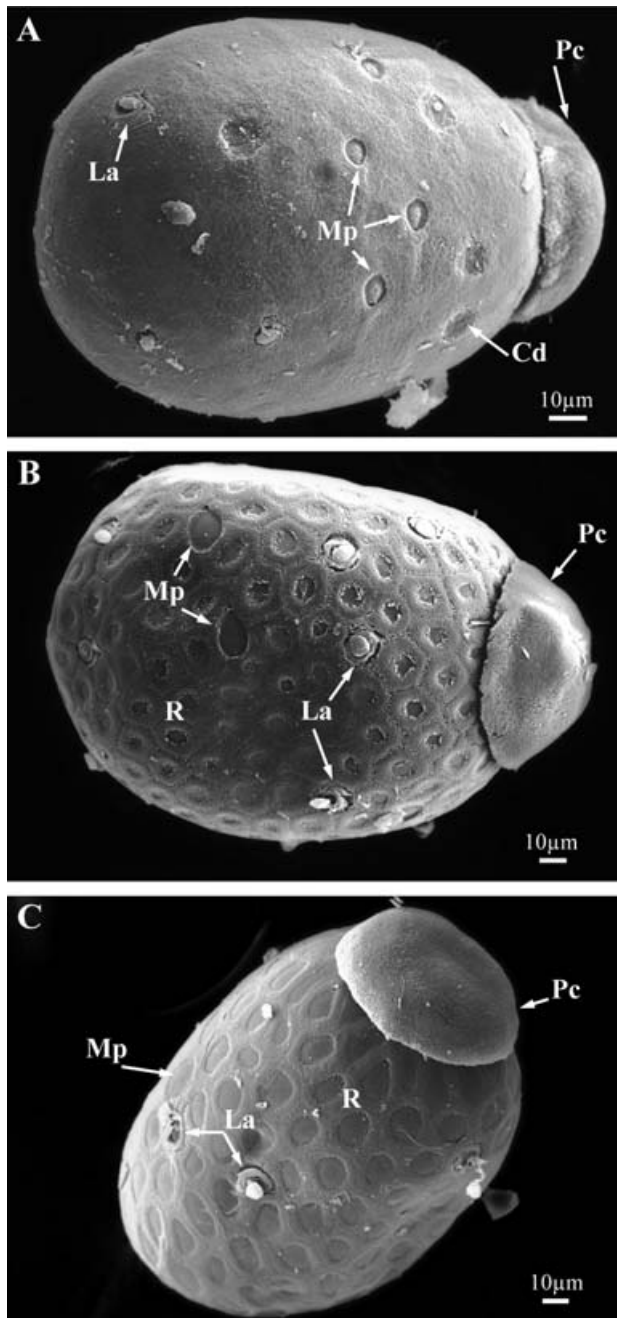


Fig. 4—Overall view of the eggs of the studied species: —**A.** *S. ignita*. —**B.** *S. spinosa*. —**C.** *T. major*. Cd, circular depression; La, lateral attachment structure; Mp, micropyle; Pc, polar cap; R, reticulation.

This winding is much more evident in the polar cap of the eggs of *Serratella* Walsh, 1862 than of *T. major* (Fig. 5B–D). In the former therefore we can speak of a type III polar cap (‘Multi-threaded coiled cap’), while in *T. major* the polar cap is more like type I (‘Non-coiled, single unit cap’). The shape and

size of the polar cap in *S. ignita* vary considerably (Fig. 5F), and all three types described by Gaino and Bongiovanni (1992) were observed: short, bulging, and long; in *S. spinosa* and *T. major*, on the other hand, the same two parameters remain quite stable. The polar cap is bell-shaped in *S. spinosa* and flat in *T. major* (Figs 4B and 5C).

The micropyles are usually set around the central area of the egg, occupying both the equatorial and subpolar regions (Fig. 4), although, occasionally, they may be more nearer the poles. The sperm guide and micropylar opening are the only parts of the micropyle visible by SEM, since the micropylar canal is completely internal (Fig. 6). The micropylar opening, a simple orifice at the beginning of the micropylar canal, shows no morphological variations in the three species studied (Fig. 6A,E,F); the only point to note is that in *T. major* it is situated below the mesh strand (Fig. 6F). The sperm guide, on the other hand, clearly differs both in shape and size. In *T. major* it is completely circular (Fig. 6F), while in *S. ignita* and *S. spinosa* it is oval (Fig. 6A,C,E). In turn, in *S. spinosa* this structure is teardrop-shaped (version of the oval shape) where the proximal end is narrow and drawn out before the micropylar canal begins (Fig. 6E). This type of sperm guide also occurs in *S. ignita* (Fig. 6C), although less frequently and in combination with the oval shape (Fig. 6B). As with the lateral attachment structures, the dimensions of the sperm guide vary between the species studied, and follow the same tendency, i.e. in *S. ignita* they are smaller (even the tear-shape) than in *S. spinosa* and *T. major* (Table 2). The number of micropyles in the egg also varies (Table 2), although, in this case, *T. major* has the lowest number per egg.

The chorionic sculpturing differs considerably among the three species (Fig. 4), despite the fact that the eggs of *S. spinosa* and *T. major* share a reticular chorionic pattern. The chorionic sculpturing of each species is characterized as follows:

- *Serratella ignita*. The chorion appears to be completely smooth (Fig. 4A), but above 10,000x irregular shallow furrows can be observed, from which arise small protuberances about 0.35 µm thick (Fig. 7A). These protuberances tend to lean sideways and to be joined to each other, although they are not visible when the extrachorion is present.
- *Serratella spinosa*. The chorion forms a geometric macrorelief formed of a large-meshed reticulation covering the whole egg surface (Fig. 4B), although nearer to the poles they may lose their regular appearance and present other geometric shapes. Each mesh unit is perfectly delimited by a very narrow furrow approximately 1.3 µm wide, which makes up the strand. The perimeter of each mesh unit is raised (3.9–2.0 µm high) and surrounds a central crater of varying diameter (8.6–4.5 µm). The external edge of the raised perimeter keeps the polygonal shape of the mesh unit, while the internal edge completely loses this shape. The size of the mesh units is quite homogeneous

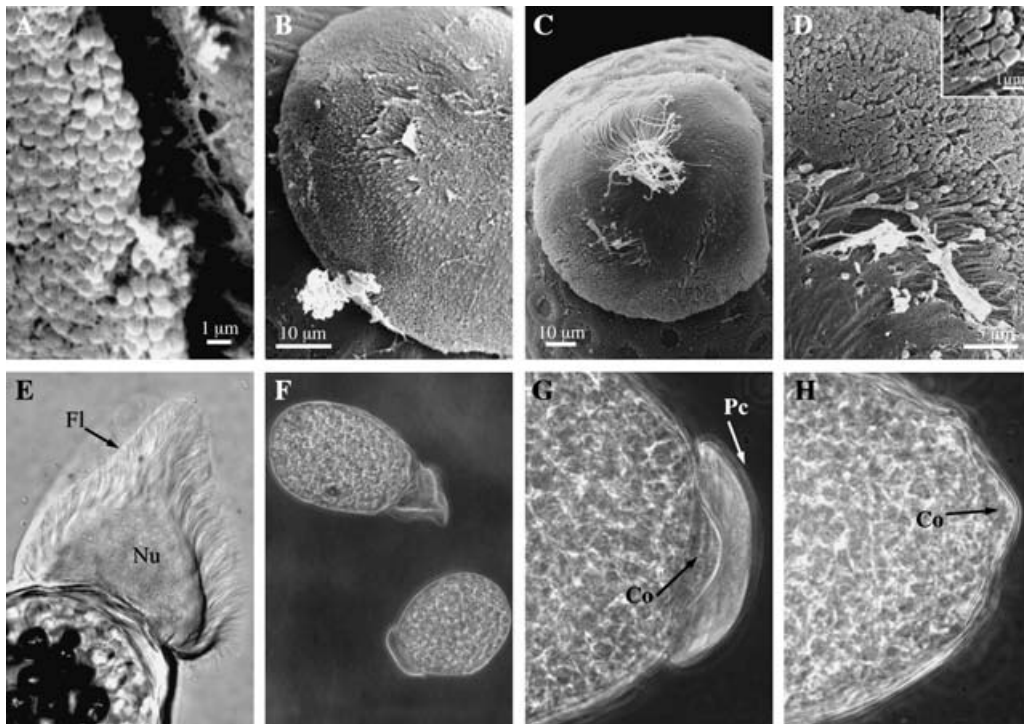


Fig. 5—Morphology and organization of polar cap. —**A, D**. Details of bulbous end in filaments. —**B, C**. General disposition of filaments in polar cap. —**E**. Internal organization of polar cap. —**F**. Varying of size and shape of polar cap. —**G**. Polar chorionic structure with polar cap attached. —**H**. Polar chorionic structure with no polar cap. *T. major* (A, B and H); *S. spinosa* (C and D); *S. ignita* (E, F and G). Light microscopy scale: 40× (E, G and H); 10× (F). Co, conical projection; Fl, filament; Nu, nucleus; Pc, polar cap.

regardless of the geometry to the individual cells (Table 3). The presence of an extrachorion does not obscure the chorion sculpturing, although it might impede observation of the strand.

- *Torleya major*. The chorion forms a geometric macrorelief constituted by a large-meshed reticulation (Fig. 4C) that extends over the whole egg surface except the polar zones, although it is more evident in the pole without a cap (Fig. 7D). Normally, the mesh units are not delimited by a furrow (Fig. 7C), so that the raised area of the chorion (6.3 μm wide) constitutes the strand of the reticle and the circular depressions (11.5 μm diameter) the mesh (Fig. 7C). At very large magnification, a very weak furrow can just be made out and the cells are seen to have a regular hexagonal shape (Fig. 7C), except near the poles. The presence of the extrachorion does not obscure the chorionic sculpturing.

Discussion

Among Ephemeroptera, the egg morphology of Ephemerellidae has been the most widely studied, at least with regard to the number of species. However, SEM descriptions tend to be very general or contain little detail, as is the case of

S. spinosa and *T. major* (Studemann *et al.* 1995; Studemann and Landolt 1997). An exception to this is *S. ignita* since both the regional and radial complexity of the chorion is well known from light microscopic (Degrange 1960) and SEM studies (Gaino and Bongiovanni 1992). Despite this, our study of the above species has provided new and surprising data, especially with regard to the lateral attachment structure.

Multithread-folded with terminal fibers cluster

The fibrous nature of the lateral attachment structures in the eggs of several species of Ephemerellidae was described by Bengtsson (1913) and Smith (1935). Much later, Degrange (1960) described their structure and organization in detail. Later, Koss and Edmunds (1974) confirmed these findings and included them in the KCT type, alongside the lateral attachment structures observed in the eggs of other families of Ephemeroptera, such as Heptageniidae, Siphonuriidae, Potamanthidae and Oligoneuriidae. Given that the description and classification of lateral attachment structures in Ephemeroptera provided by Koss and Edmunds (1974) are considered the main reference in chorion morphological studies in these insects, SEM-based morphological studies of the eggs of the family Ephemerellidae have continued to

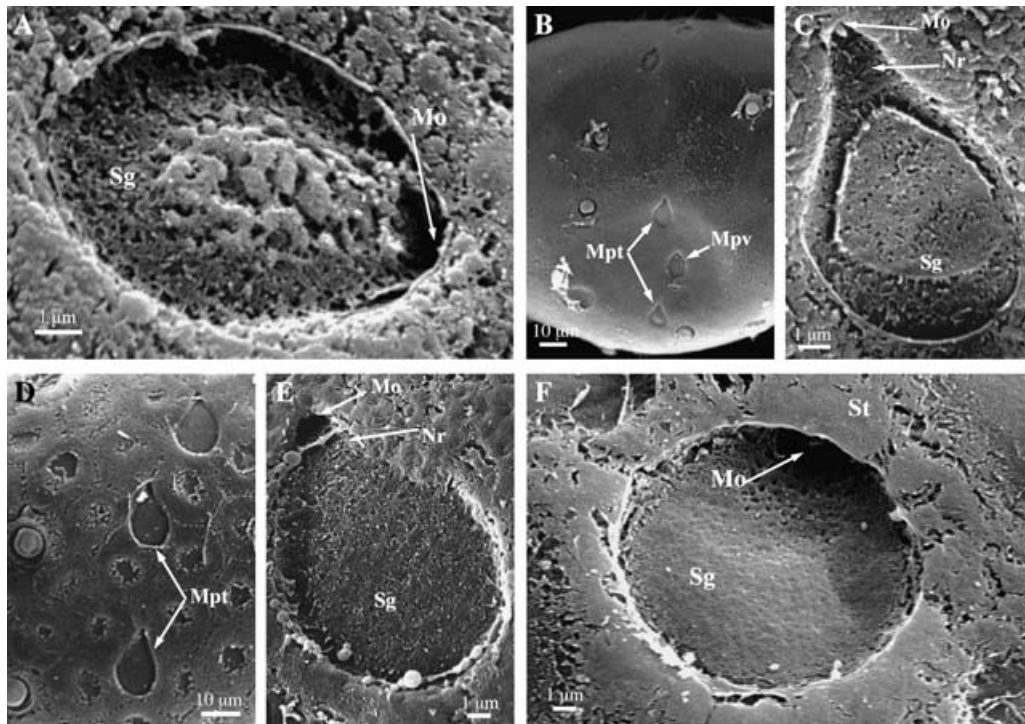


Fig. 6—Sperm guide morphology and micropyles distribution in eggs. —**A**. Oval sperm guide. —**B**. Distribution of micropyles with both teardrop-shaped and oval sperm guides visible. —**C**, **E**. Teardrop-shaped sperm guide. —**D**. Distribution of micropyles in the reticulation. —**F**. Circular sperm guide. *S. ignita* (A and C); *S. spinosa* (D and E); *T. major* (F). Mo, micropylar opening; Mpt, teardrop-shaped micropyle; Mpv, oval-shaped micropyle; Sg, sperm guide; St, strand of reticulation.

regard lateral attachment structures as KCT (Gaino and Bongiovanni 1991, 1992; Kang and Yang 1995; Studemann *et al.* 1995; Studemann and Landolt 1997). However, as in the case of the lateral attachment structures of the eggs of *Potamanthus luteus* (Linnaeus, 1767) (Potamanthidae), in which SEM studies revealed morphological characteristics that cannot be considered KCTs (Ubero-Pascal and Puig 2007), the morphological characteristics of the lateral attachment structures of Ephemerellidae eggs also lead us to question the type to which they have traditionally been assigned.

The KCT consists of a thread composed of several twisted filaments that support a terminal knob (Koss and Edmunds 1974; Gaino and Bongiovanni 1991). Recently, Gaino and Reborá (2003) made a detailed study of the morphology and organization of KCTs using SEM and TEM in the species *Ecdyonurus venosus* (Fabricius, 1775) (Heptageniidae), whereby these observations were confirmed and new interesting information as regards the way in which eggs are fixed to the substrate was provided. Comparison of the SEM description of a KCT given by these authors and our observation of lateral attachment structures in the eggs of the three species of the Ephemerellidae studied, suggest a totally different ultrastructure and packing arrangement. This arrangement consists of a fibrous ultrastructure formed of threads, each

made up of a few weakly twisted filaments that, proximally, are fixed to the chorion at diametrically opposite sites of the edge of a circular depression and, distally, to a multifibrillar circular cushion. The whole structure is packed within the depression by multiple zigzag foldings of threads, leaving the cushion in a central position. This description clearly differentiates the structure observed from a KCT and any other structure proposed by Koss and Edmunds (1974). Bearing this in mind, we propose a new lateral attachment structure, provisionally denominated MFT. In this way and with regard to their organization and packing, the fibrous attachment structures defined by Koss and Edmunds (1974) could be classified as noncoiled, coiled and folded.

Although the morphological characteristics of the lateral attachment structures in Ephemerellidae species have not been described previously by SEM, analysis of the photographs that have been published clearly points to some of the characteristics we describe above, both in the species that we have studied (*S. ignita*, *S. spinosa* and *T. major*) and in another 12. Therefore, we propose that the following species should be considered as having MFT type lateral attachment structures: *Ephemerella oroni* Eaton, 1908 (cited as *Ephemerella aurivillii* Bengtsson, 1908), *Drunella submontana* (Brodsky, 1930), *Drunella colouradensis* (Dodds, 1923), *Drunella doddsi*

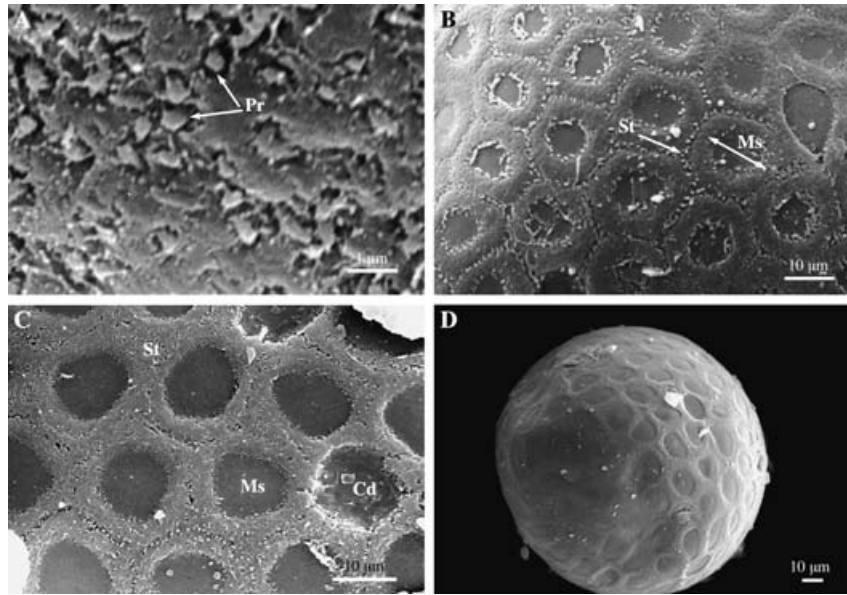


Fig. 7—Chorionic sculpturing.
 —**A.** *S. ignita*, detail of chorion surface.
 —**B.** *S. spinosa*, reticular geometric macrorelief.
 —**C.** *T. major*, reticular geometric macrorelief.
 —**D.** *T. major*, detail of the pole opposite the polar cap showing absence of sculpturing. Cd, circular depression; Ms, mesh of reticulation; Pr, protuberance; St, strand of reticulation.

(Needham, 1927), *Drunella paradinasi* González del Tánago and García de Jalón, 1983, *Acerella glebosa* Kang and Yang, 1995, *Acerella montana* Kang and Yang, 1995, *Cincticostella colossa* Kang and Yang, 1995, *Cincticostella fusca* Kang and Yang, 1995, *Eburella brocha* Kang and Yang, 1995, and *T. lutosa* Kang and Yang, 1995 (figs 50, 52 and 54 in Studemann and Landolt 1997; figs 2, 5 and 11 in Studemann *et al.* 1995; figs 8, 10, 12, 15, 19 and 23 in Kang and Yang 1995). Although the photographs published for other species in these and other studies (see Jacobus and Sartori 2004; Jacobus *et al.* 2004) do not permit us to affirm that these structures are MFT type, there seem to be strong indications that they should be considered as such. However, we think that the family Ephemerellidae only has MFT type lateral attachment structures, with the exception of the genus *Eurylophella* Tiensuu, 1935 which has no lateral attachment structure (Studemann and Landolt 1997). From a systematic point of view, this would characterize this family since such structures have not been observed in any other family (Ubero-Pascal 2004).

Comparison of egg morphology

The shape and overall appearance of the eggs of the species studied fit the most common morphotype of the family Ephemerellidae, being ovoid, having a polar cap, numerous micropyles and lateral attachment structures. In agreement with Studemann *et al.* (1995) and Studemann and Landolt (1997), no intraspecific variations of the chorionic pattern were seen between the species studied, even in the case of *S. ignita*, whose eggs come from both nymphs and female imagos. However, the mean size of the eggs studied strongly

contrasts with the values found in the literature, since our measurements are up to 100 μm less than those mentioned for *S. ignita* and *S. spinosa* (Studemann *et al.* 1995). Egg size may be influenced by biological factors (life cycles, feeding, relationships between size of female abdomen and egg number, etc.) and by the way in which they are handled in the laboratory (fixation, preservation, preparation for microscope study, etc.) (Degrange 1960; Soldan 1979; Brittain 1982; Studemann and Landolt 1997; Ubero-Pascal *et al.* 2005). Possibly the fact that unfixated eggs were studied may explain the values obtained by Degrange (1960), as has been confirmed in other species of Ephemeroptera (Ubero-Pascal and Puig 2007), while any of the above mentioned factors could explain the variation from the values given by Studemann *et al.* (1995) and Gaino and Bongiovanni (1992), including the way in which they were measured. Therefore, egg size should be treated with caution in comparative morphological studies since many factors may interfere.

Since Koss and Edmunds (1974) indicated that the polar cap of Ephemerellidae eggs showed type I characteristics (*noncoiled, single unit cap*), all subsequent studies, including SEM studies, have followed this classification (Kang and Yang 1995; Studemann *et al.* 1995; Studemann and Landolt 1997), except the study carried out by Gaino and Rebora (1992) in *S. ignita*. These authors considered that the filaments of the polar cap were curled into a spiral in the same way as the polar cap of the eggs of *Caenis* Stephens, 1835 (Caenidae) (Malzacher 1982), although more compact and much less spiralled, which made them like Koss and Edmunds' type III. Our observations not only confirm the type III cap in *S. ignita*, but also extend the type to *S. spinosa*. In contrast, the spiral disposition of the filaments of the polar

cap of *T. major* is not so clear when observed by SEM, although it is just possible to see it by light microscopy. The SEM study made by Kang and Yang (1995) shows the loosely spiral nature of the filaments in other species of *Torleya* Lestage, 1917, although this is not specified in their description, while in other genera (*Cincticostella* Allen, 1971 and *Eburella* Kang and Yang, 1995) the filaments of the polar cap are clearly type I. It is evident, then, that both types of polar cap can exist in the family Ephemerellidae.

Although the shape and size of the polar cap is more or less constant in Ephemerellidae eggs, some species present considerable variability in these parameters (Studemann and Landolt 1997). In *S. ignita* this variability has been mentioned in several studies (Bengtsson 1913; Degrange 1960; Gaino and Bongiovanni 1992; Studemann *et al.* 1995), and is confirmed by our observations. On the other hand, in contrast to Studemann and Landolt (1997), we did not find this variability in *S. spinosa*. The variation in egg polar cap length and shape of some species of Ephemeroptera means that these characteristics should be treated with care if used for taxonomic purposes (Gaino and Bongiovanni 1992; Ubero-Pascal *et al.* 2005; Ubero-Pascal and Puig 2007). Our studies confirm the presence of a chorionic structure in the form of a circular plate in the eggs of the family Ephemerellidae (already mentioned by Degrange 1960), over which the filaments of the polar cap are disposed. Furthermore, the shape of the end swellings of the cap filaments may also change, as seen in the differences between *S. ignita* and *S. spinosa*, although a more exhaustive study is necessary before any significant variability can be described.

The micropyles in the eggs of the family Ephemerellidae are tageniform, with a completely interiorized micropylar canal, and only the sperm guide is visible by SEM. The shape of the sperm guide is usually oval or ovoid in the eggs of the family Ephemerellidae (Studemann *et al.* 1995; Studemann and Landolt 1997; Jacobus *et al.* 2004), although they may be more circular in some species such as *T. major*. A teardrop-shaped sperm guide, as well as the oval form, is quite common in species of Ephemerellidae (Kang and Yang 1995; Studemann and Landolt 1997), but in *S. spinosa* may be the only type. The number of micropyles in the egg varies with species within the family Ephemerellidae but, as with egg size, there were substantial differences between our findings and the data found in the literature for *S. spinosa*. For example, we counted a maximum of five on the half egg visible by SEM, while Studemann *et al.* (1995) found up to 8 or even 12, although it is unclear whether this range corresponds to those visible on half an egg or whether it is an estimate of the total number of micropyles per egg, which is what we refer to. Different observations in eggs with multiple micropyles, both in species belonging to Ephemerellidae and other families of Ephemeroptera, lead us to think that the distribution around the egg is not totally uniform and that they are probably grouped on one side of the egg. Therefore, any extrapolation of the number of micropyles from the

number observed by SEM on half an egg must be made with caution.

Chorionic sculpturing shows great variation in the eggs of Ephemerellidae, although, in agreement with the observations of Studemann and Landolt (1997), this variation can be reduced to two basic patterns: apparently smooth eggs with a uniform chorion, as in *S. ignita*, and eggs with a geometric macrorelief, as in *S. spinosa* and *T. major*. The ornamentation of the species *Eurylophella* and *Timpanoga* Needham, 1927 fit this last pattern. Eggs with a geometric macrorelief are found in 10 genera, while apparently smooth eggs are found in four (Kang and Yang 1995; Studemann and Landolt 1997; Jacobus and Sartori 2004; Jacobus *et al.* 2004). The great variability in the morphotype of eggs with a geometric macrorelief means that chorionic sculpturing in this family can be considered species-specific (Kang and Yang 1995; Studemann *et al.* 1995). However, the characteristic found on the chorionic surface of *S. ignita* eggs, which have a uniform and smooth morphotype, suggests that specific characteristics may occur in other species of the same morphotype, so that a more detailed study is necessary. In addition, the absence of any sculpturing at the pole opposite the polar cap in *T. major* seems to be shared by other species of the genus *Torleya* (Kang and Yang 1995; Jacobus *et al.* 2004).

Morphological studies of Ephemerellidae eggs have basically been put to systematic ends, either from a purely taxonomic point of view (Degrange 1960; Studemann *et al.* 1995) or phylogenic (Studemann and Landolt 1997), in an attempt to resolve the problems inherent in classifying this family. However, such attempts have not been very successful to date; furthermore, McCafferty and Wang (1994) did not consider egg morphology to be of much use in a phylogenetic study of the *Timpanoga*-complex since little information was available for some species. They considered that a deeper study of egg structures was needed and that the egg morphology of Ephemeroptera is subject to a degree of convergence. Studemann *et al.* (1995) and Studemann and Landolt (1997), on the other hand, concluded that egg morphology was useful taxonomically at species level although it does not serve much purpose for characterizing genera. Despite the wealth of studies on the egg morphology of this family, our contribution provides new information, including the type of lateral attachment structure, which seems only to occur in this family (Ubero-Pascal 2004). It is necessary, then, to study egg morphology in greater depth even in those eggs that are well known, as well as in other species, to confirm our findings and to ascertain whether they can be useful in systematics. With greater knowledge of the organization of the polar cap, the appearance of the bulbous end of the filaments, micropyle morphology or the characteristics of the chorion surface (probably obtained by greater magnification), it should be possible to resolve the problems of identification posed by Studemann *et al.* (1995) between the eggs of *E. aurivillii* and *E. ignita*, on the one hand, and those of *T. major* and *D. paradimasi* on the other.

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