New Application of Air-Drying Techniques for Studying Ephemeroptera and Plecoptera Eggs by Scanning Electron Microscopy

NICOLÁS UBERO-PASCAL,[1]* JOSÉ MANUEL FORTUÑO,[2] AND MARÍA DE LOS ÁNGELES PUIG[3]
[1]*Departamento de Zoología y Antropología Física, Facultad de Biología, Universidad de Murcia, Campus de Espinardo s/n, 30100 Espinardo-Murcia, Spain*
[2]*Institut de Ciències del Mar, Centre Mediterrani d'Investigacions Marines i Ambientals-CSIC, Laboratorio de Microscopía Electrónica, Passeig Maritim de la Barceloneta, 37-49. 08003 Barcelona, Spain*
[3]*Centro de Estudios Avanzados de Blanes (CEAB-CSIC), Acces Cala Sant Francesc, 14, 17300 Blanes, Spain*

KEY WORDS egg morphology; air-drying techniques; critical point drying; hexamethyldisilizane; tetramethylsilane; SEM

ABSTRACT Hexamethyldisilizane (HMDS) and tetramethylsilane are organic compounds that are volatile at ambient temperature and which can therefore be used for air-drying biological samples for SEM studies. The techniques using these compounds provide results that are comparable with those obtained by critical point drying, but which involve a very simple process that saves time and money. Both techniques were applied to SEM studies of Ephemeroptera and Plecoptera eggs in order to assess their suitability as alternative methods to critical point drying for these kinds of biological material. The results show no morphological differences between eggs HMDS air-dried and critical point-dried. *Microsc. Res. Tech. 68:264–271, 2005.*

INTRODUCTION

The eggs of Ephemeroptera and Plecoptera possess numerous chorionic features of great taxonomic and systematic interest (Domínguez and Cuezzo, 2002; Klonowska, 1997; Koss and Edmunds, 1974; Stark and Szczytko, 1982, 1988; Studemann and Landolt, 1997; Studemann and Tomka, 1991). Although some of these features have already been described by optical microscopy techniques (Bengtsson 1913, Degrange 1960, Hynes, 1941, 1974; Koss, 1968; Koss and Edmunds 1974, Smith, 1935), scanning electron microscopy (SEM) has revealed their great variety and allowed detailed descriptions of the external morphological organization of the chorion in these insects (Gaino and Bongiovanni, 1992, 1993; Gaino and Mazzini, 1987; Gaino et al., 1987, 1989; Isobe, 1997; Klonowska, 1997, Klonowska and Jazdzewska, 2003; Kopelke and Müller-Liebenau, 1981a,b, 1982; Stark and Szczytko, 1982, 1988; Ubero-Pascal, 2004; Ubero-Pascal et al., 2001).

The procedure for preparing biological samples for SEM analysis involves several progressive stages (Goldstein et al., 1992); drying is a very delicate operation, since the surface tension that may be produced can result in distortions in the shape and general appearance of eggs. A deficient drying of eggs, even after proper fixation and dehydration, can cause shrinking or collapse and/or the appearance of structural artifacts in their chorionic surface, such as wrinkles, cracks, depressions, etc. (Ubero-Pascal, 2004). Such deformations can induce the incorrect interpretation of the external morphology, because not only do they prevent the correct observation of form and size, but they also mask the morphology of some of the chorionic features.

Most SEM studies on Ephemeroptera and Plecoptera eggs use the critical point drying technique because of the excellent results obtained for chorionic morphology (Domínguez and Cuezzo, 2001; Gaino and Bongiovanni, 1992, 1993; Gaino and Mazzini, 1987; Gaino and Rebora, 2001; Gaino et al., 1987; Isobe, 1997; Klonowska, 1997; Mazzini and Gaino, 1985; Studemann and Tomka, 1991). However, this technique has its disadvantages, including the time-consuming nature of the process and the complex equipment necessary, which increases the cost, especially when there are numerous samples to analyze. Other drying techniques which take advantage of the hardness of the egg chorion, such as direct air-drying (Kopelke and Müller-Liebenau, 1981a,b, 1982, Rościszewska, 1996; Stark and Szczytko, 1982) or drying in a stove (Tierno de Figueroa, 1998), are simple procedures, which reduce the time and cost involved. However, the results are sometimes poor, since the eggs may break or be greatly deformed, especially in the case of Ephemeroptera eggs

Hexamethyldisilizane (HMDS) and tretramethylsilane (TMS) are two organic compounds, characterized by their good miscibility with standard chemicals used for fixing and preserving biological samples and by their low surface tension, which results in their

*Correspondence to: Dr. Nicolás Ubero-Pascal, Departamento de Zoología y Antropología Física, Facultad de Biología, Universidad de Murcia, Campus de Espinardo s/n, 30100, Espinardo-Murcia, Spain. E-mail: nubero@um.es

Received 26 July 2005; accepted in revised form 28 September 2005
Contract grant sponsor: The TOXCIARIMED project; Contract grant number: REN2000-1021-C03-01/HID.
DOI 10.1002/jemt.20248
Published online in Wiley InterScience (www.interscience.wiley.com).

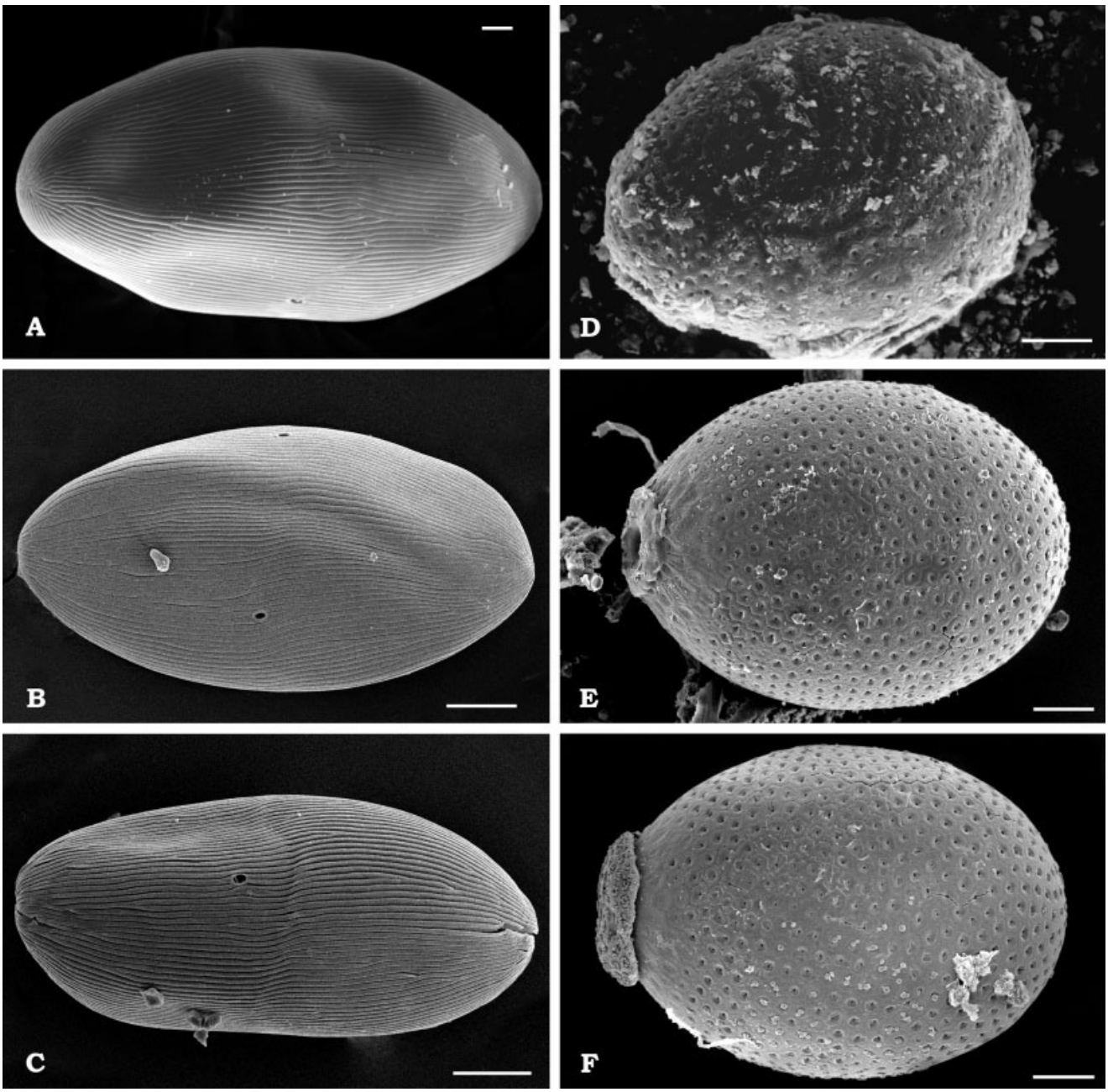

Fig. 1. Comparison of shape and general appearance in critical point dried (CPD) and HMDS/TMS-treated/air-dried eggs. **A:** *Habroleptoides nervulosa* egg, CPD; scale bar = 10 μm. **B:** *H. nervulosa* egg, HMDS air-dried, scale bar = 25 μm. **C:** *H. nervulosa* egg, TMS air-dried, scale bar = 25 μm. **D:** *Perla marginata* egg, CPD, scale bar = 50 μm. **E:** *Perla marginata* egg, HMDS air-dried, scale bar = 50 μm. **F:** *Perla marginata* egg, TMS air-dried, scale bar = 50 μm.

gradual evaporation at room temperature, characteristics which have led to their use as a pretreatment for air-drying biological samples for SEM study (Botes et al., 2002; Dey et al., 1989; Nation, 1983). These procedures have been proposed as alternatives to critical point drying for studying invertebrate soft tissue, not only because the results obtained with both techniques are similar, but also because air-drying with HMDS and TMS is faster, inexpensive and does not require complex equipment.

We applied the HMDS and TMS air-drying techniques to the SEM morphological study of Ephemeroptera and Plecoptera eggs in order to assess any advantages compared with critical point drying. The comparative results are shown in this paper.

MATERIALS AND METHODS

The studied eggs were obtained from female nymphs fixed in 4% formaldehyde and conserved in 70% etha-

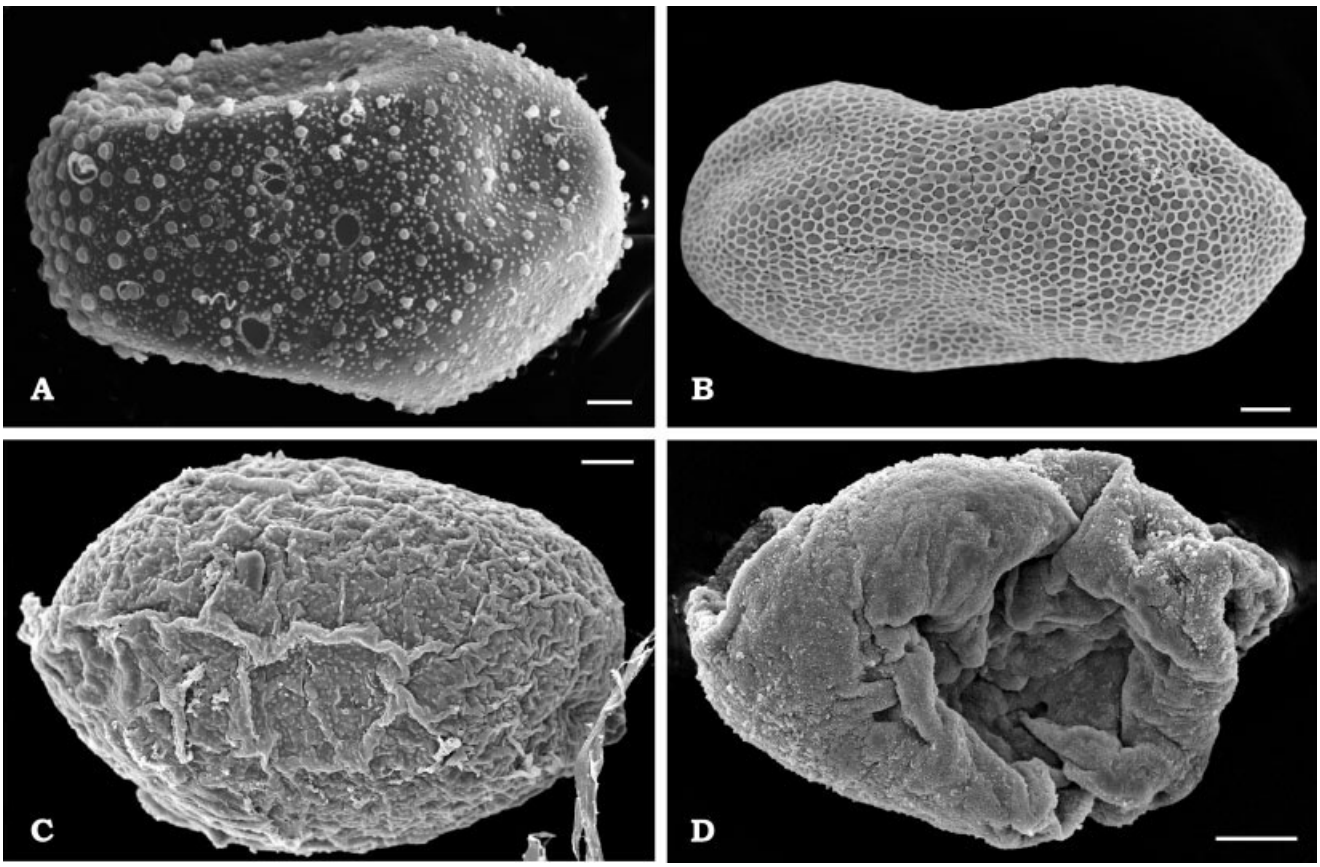

Fig. 2. Comparison of morphological distortion in critical point dried (CPD) and HMDS/TMS-treated/air-dried eggs. **A:** *Ecdyonurus focipula* egg showing large depression, CPD, scale bar = 10 μm. **B:** *Centroptilum luteolum* egg showing large depresion, HMDS air-dried, scale bar = 10 μm. **C:** *Alainites muticus* egg showing large and branched wrinkle, TMS air-dried, scale bar = 10 μm. **D:** *Cloeon dipterum* egg collapsed, TMS air-dried, scale bar = 10 μm.

nol, 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 nytal bags of 50-μm mesh, and kept in Eppendorf tubes with ethanol 70% until the beginning of the SEM preparation procedure. Any manipulation of the eggs during the different stages of the SEM preparation procedure was made in the nytal bags.

The eggs were cleaned in a Branson 3510 ultrasound bath for 5 min and dehydrated in increasing concentrations of ethanol (80%, 90%, 95%) until absolute ethanol, the samples remaining 10 min in each bath. Drying was carried out by two techniques:

a. Critical point drying: Acetone was used as intermediate liquid in this technique, with the dehydrated eggs remaining in an 50% acetone–ethanol bath for 10 min before passing to absolute acetone. Critical point drying was carried out in a Balzer-Union cpd 020 drier, using CO_2 as the transition liquid.

b. Air-drying: Absolute ethanol was used as intermediate liquid and hexamethyldisilizane (HMDS) or tetramethylsilane (TMS) as the transition liquid. The process was carried out in a laminar flow hood with the eggs placed in a glass container of TMS or HMDS. After 15 min, the bags were removed from the bath and were allowed to dry in air for 25–30 min, until evaporation of the organic compounds was complete.

After the drying stage, the nytal bags were opened under a stereoscopic microscope and the eggs were mounted, with the aid of micromanipulators, on stubs provided with double sided conductive sticky tape. In this way, the eggs were orientated in all possible directions. The eggs mounted were 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 KV and 10 KV, respectively.

To compare the different drying methods, egg morphology was studied in 37 species (28 Ephemeroptera and 9 Plecoptera), the data already published in the bibliography being included. All drying techniques were applied to specimens of 17 species (12 Ephemeroptera and 5 Plecoptera), while critical point drying and one of the air-drying methods were applied to the rest of species. For each species and method 40–50 eggs were prepared for SEM analysis, verifying the morphology of the chorion in at least 10 of them.

RESULTS

The two drying techniques were evaluated by comparing several aspects of the external morphology of

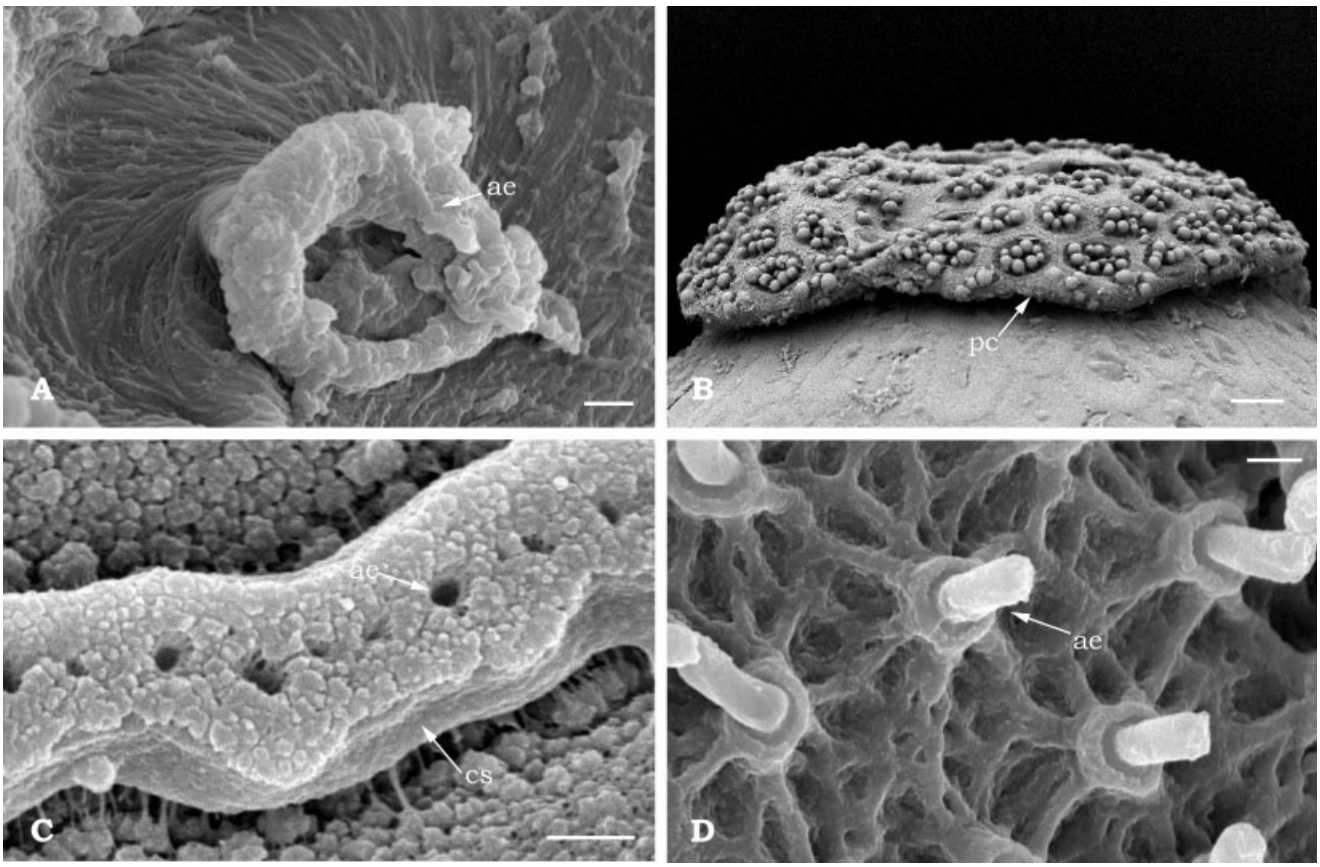

Fig. 3. Shape of different basic structures of HMDS or TMS air-dried eggs. **A:** Fibrous attachment structure (ae) in *Oligoneuriella rhenana* egg (HMDS), scale bar = 1 μm. **B:** Fibrous polar attachment structure (ae) in *Perla marginata* egg (TMS), scale bar = 10 μm. **C:** Nonfibrous attachment structure over longitudinal costae (cs) and aeropyles (ap) in *Habrophlebia fusca* egg (HMDS), scale bar = 1 μm. **D:** Nonfibrous attachment structure (ae) and irregular reticulation in *Paraleptophlebia submarginata* egg (TMS), scale bar = 1 μm.

the eggs, such as overall shape, main type of functional features (attachment structures, micropyles, aeropyles, and chorionic sculpturing) (Koss and Edmunds, 1974; Stark and Szczytko, 1988), and the presence and aspect of the extrachorion or temporary external membrane (Ubero-Pascal, 2004; Ubero-Pascal et al., 2001).

Both the critical point-dried eggs and those air-dried following treatment with HMDS or TMS generally showed a well-conserved shape and general appearance, and no substantial difference was observed between one technique and the other (Fig. 1). In some cases, slight deformations (depressions or dents affecting a large area of the egg) or artifacts (irregular branching cracks and wrinkles) were observed, although these did not affect the interpretation of the general shape of the eggs (Fig. 2a–2c). The depressions or dents did not seem to be clearly related with either drying processes, since they appeared independent of the technique or organic compound used. However, the artifacts were related to a specific drying technique, since they usually appeared in air-dried TMS-treated eggs. Total collapse of the eggs was only observed in *Cloeon dipterum*, although this may well have been more related with the thinness of the chorion, given its ovoviviparity, rather than with the drying technique used (Fig. 2d).

The structural morphology and organization of the main functional features of the egg chorion were apparently unaffected by the drying technique used, since all the parts were observable and there were no notable differences between the critical point- and air-dried eggs (Figs. 3 and 4). Usually no type of deformation or artifact that could be related to the drying techniques was detected in these kinds of structures.

As with critical point drying, air-drying after treatment with HMDS or TMS allowed the attachment structures to be clearly differentiated (Fig. 3) into fibrous, granular, or indistinguishable. The air-drying technique also allowed detailed study of the parts or constituent elements of the attachment structures, micropyles (sperm guide and micropylar canal) (Figs. 4c,d and 6b,d), aeropyles, (Figs. 3c and 4b,d) and chorionic sculpturing (Figs. 3 and 4), including its different morphologic types. In the case of chorionic sculpturing, structures such as the mesh (Figs. 3d and 4b–d) and micromesh (Fig. 4a), ribs (Fig. 3c and 4a), different types of protuberance (Figs. 3d and 6b), etc., were easily distinguishable.

The extrachorion covering the whole egg was easily observed in air-dried eggs, where it was in very close contact with the chorionic surface, and maintained its transparency (Figs. 5c and 6a), whereas in the critical

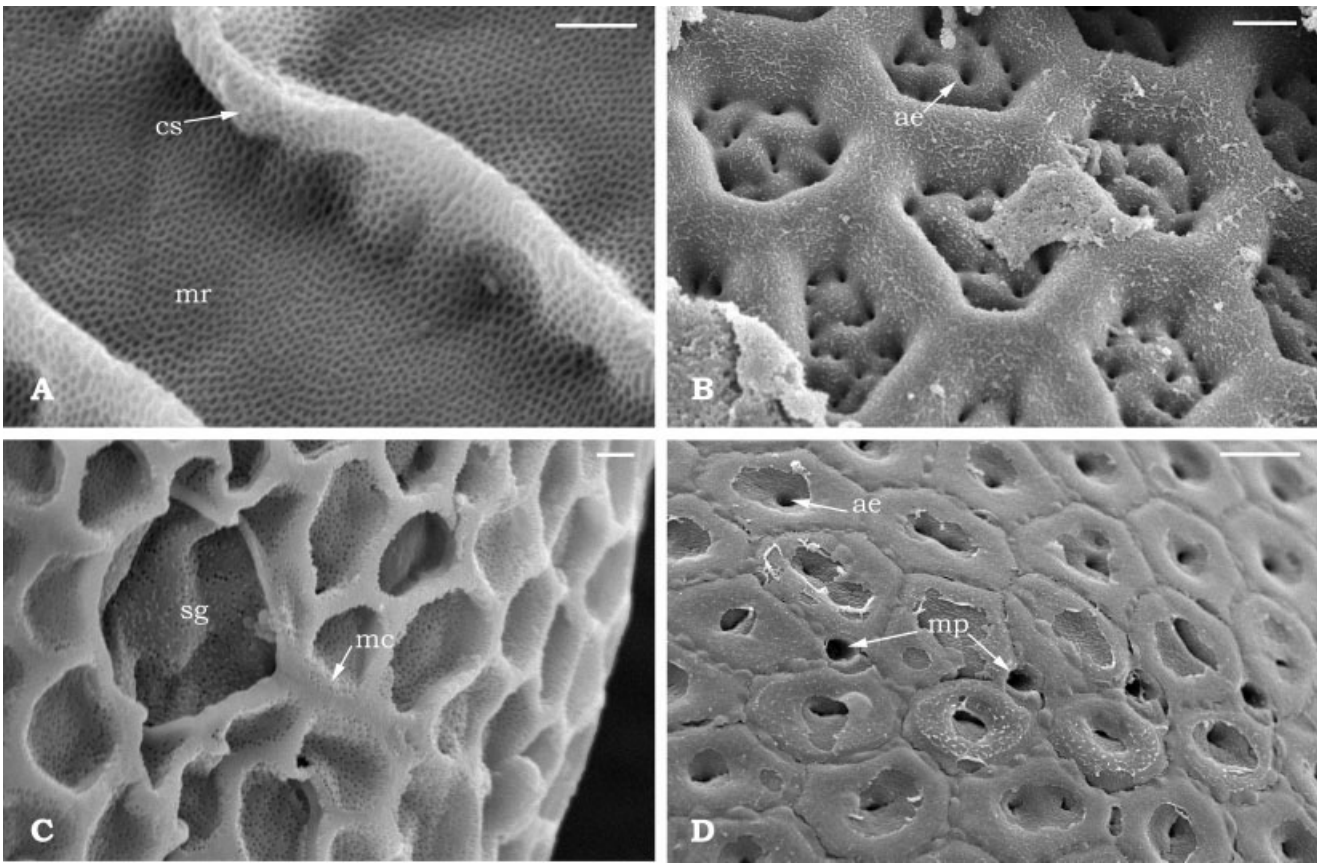

Fig. 4. Shape of different basic structures of HMDS or TMS air-dried eggs. **A:** Longitudinal costae (cs) and microreticulation (mr) in *Acenterlla almohades* egg (HMDS), scale bar = 1 μm. **B:** Reticulation and aeropyles (ap) in *Isoperla grammatica* egg (HMDS), scale bar = 5 μm. **C:** Reticulation and micropyle (sg = sperm guide, mc = micropilar canal) in *Centroptilum luteolum* egg (TMS), scale bar = 1 μm. **D:** Reticulation, aeropyles (ap) and micropyles (mp) in *Perla marginata* egg (TMS), scale bar = 10 μm.

point-dried eggs, the extrachorion sometimes appeared detached (Figs. 5a,b and 6b,d) and opaque (Figs. 5c,d). In *Ephemera danica*, the extrachorion completely covered the eggs, constituting a thick envelope, although they had been critical point-dried (Fig. 6c).

DISCUSSION

The process of air-drying eggs after HMDS/TMS treatment is considerably faster and simpler than critical point drying (Dey et al., 1989 ;Nation, 1983). We have verified that a large number of samples (>10), each containing a substantial number of eggs (>50), can be dried in 5 min at most, meaning that they can be mounted on stubs and metallized in a single morning's work. Critical point drying on the other hand, requires at least one and half hours for each sample, meaning approximately 15 h for 10 samples, without counting the time needed to mount on stubs and metal coating. This saving in time is mainly due to the fact that all the samples can be treated with HMDS/TMS simultaneously before drying during the same work session. Therefore, the longest time is that needed for mounting the eggs on stubs prior to Au–Pd coating, a process that is, in any case, also necessary with critical point, where the work would involve successive days. In addition to the time saved, and in agreement with Nation (1983), the HMDS/TMS air-drying technique represents an economic method, since no complex equipment or specialized technician is required.

Air-drying after treatment with HMDS or TMS provided the same good results as critical point drying in the SEM morphological study of Ephemeroptera and Plecoptera eggs, as already described by Nation (1983) and Dey et al. (1989) for different invertebrate soft tissues. This drying technique allowed the interpretation and determination of egg shape and general appearance, as well as the detailed morphological description of the different structures. The same reliability and precision can be attained as with critical point, even at great magnification and using a low working voltage.

In general, the deformations and artifacts observed in the studied eggs did not seem to be related with the drying technique used, since they appeared in both critical point-dried and HMDS/TMS-treated air-dried egg. It does not seem that the previous stages of the egg preparation procedure for SEM study had any effect on this finding, since the same techniques were used for all the eggs. The origin of these distortions, therefore, must lie in the way in which the eggs are

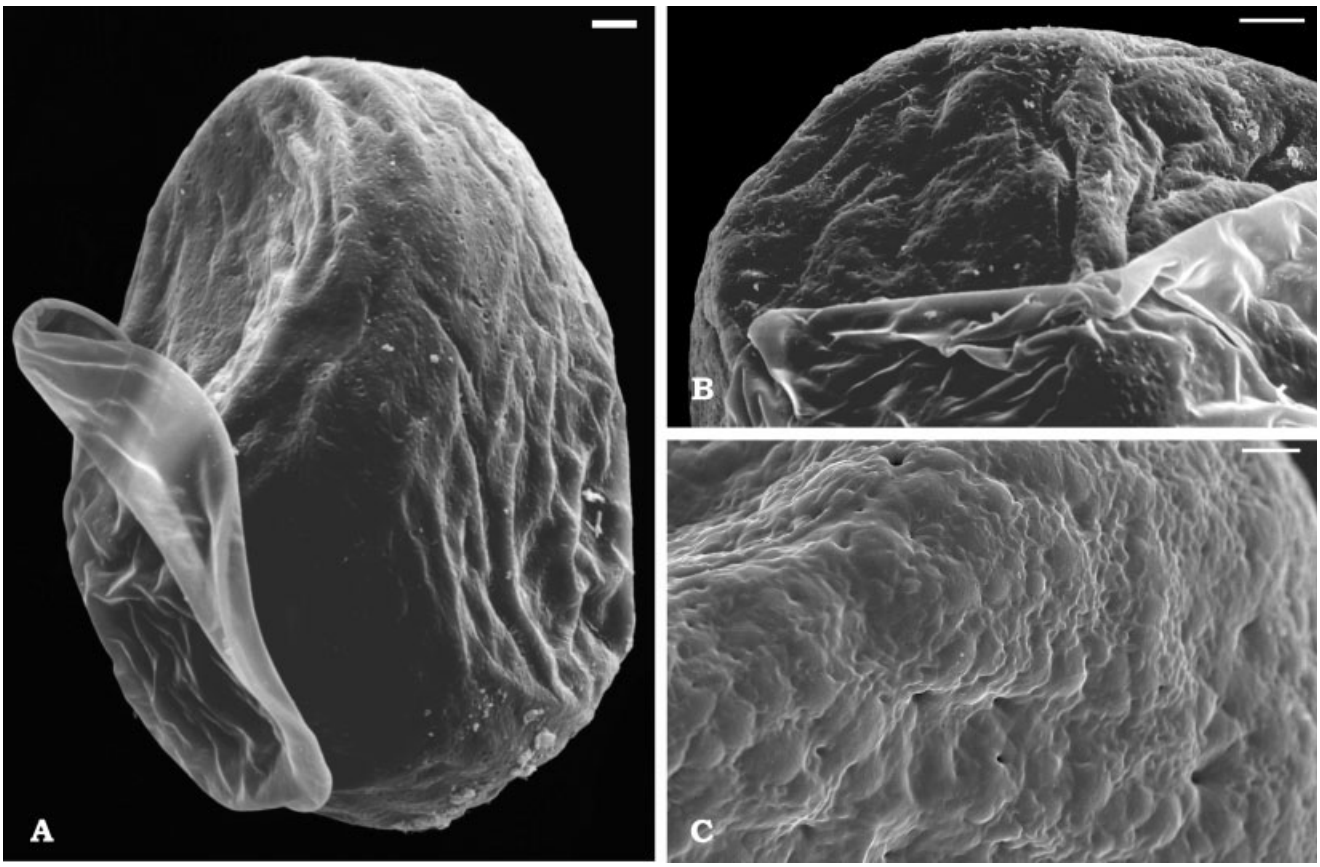

Fig. 5. Comparison of shape and disposition of extrachorion in critical point dried (CPD) and HMDS/TMS air-dried eggs of *Nemoura fulviceps*. **A:** Extrachorion completely removed (CPD), scale bar = 10 μm. **B:** Extrachorion partially removed (CPD), scale bar = 10 μm. **C:** Extrachorion fully covering the egg (HMDS), scale bar = 5 μm.

formed (Stark et al., 1987) or, in the particular case of depression or dents, in the way in which eggs are stored in the oviduct. The reduced volume of oviduct in relation to the size and number of eggs in many species may cause them to be squeezed to adapt to the space available. These deformations would disappear in natural conditions when the eggs are laid in water (Soldan, 1979).

However, the appearance of wrinkles could well be associated with the type of drying procedure used, since they were only observed in some of the eggs air-dried after treatment with TMS. However, even in this case, it cannot be ruled out that these aberrations are favored by the radial complexity of the eggs (the structural organization and thickness of the chorion layers), because the species whose eggs are more likely to present these deformations have quite thin and weak chorions, as happens in some genera of the Baetidae family (Gaino and Rebora, 2005). The description in some papers of this kind of chorionic structure, which we regard as aberrant, in eggs of different species of *Baetis*, which have been air-dried directly after removal from the preserving chemical (Kopelke and Müller-Liebenau, 1981a,b, 1982), led us to think that TMS may cause a similar behavior. TMS evaporation is faster and not as gradual as that obtained with HMDS or CO_2 in critical point drying; besides, it causes substantial hardening of the egg, meaning greater surface tension in those eggs that have a thin or structurally simple chorion, inducing the appearance of wrinkles. These circumstances might explain the wrinkled appearance and complete collapse observed in *Cloeon dipterum* eggs, since they have a thin chorion of low radial complexity (Gaino and Rebora, 2005) and were TMS air-dried. In addition, these drastic distortions might also have been favored by the presence of completely formed larvae inside the eggs.

The drying technique used did not influence the observance of an extrachorion, although it could have modified its disposition around the egg and its relation with the chorionic surface. The extrachorion on critical point-dried eggs shows a very variable morphology, even within the same species, where it may be absent, cover the egg partially, be detached, or have lost its transparency. However, to confirm the possible cause–effect relationship between critical point drying and the properties and disposition of the extrachorion, it would be necessary to previously test the HMDS/TMS air-drying technique in eggs dehydrated up to absolute acetone, since this compound may well be involved in modifying the extrachorion.

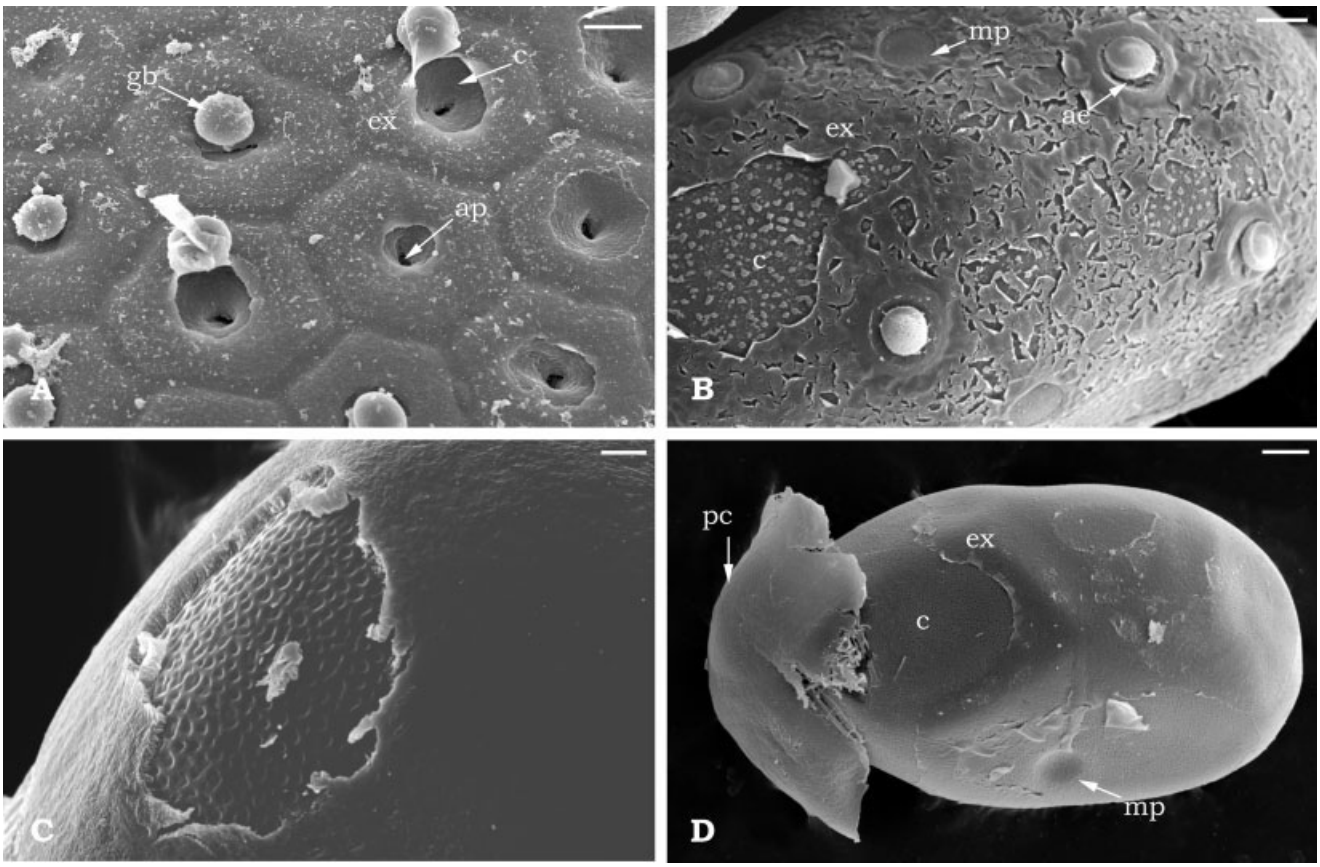

Fig. 6. Different shape and disposition of extrachorion in air-dried and critical point dried (CPD) eggs. **A:** Extrachorion (ex) fully covering the egg in *Perla marginata* (TMS) (ap = aeropyle, c = chorion, gb = globular bodies), scale bar = 5 μm. **B:** Extrachorion (ex) covering the egg but partially raised and cracked in *Potamanthus luteus* (CPD) (ae = attachment structure, c = chorion, mp = micropyle), scale bar = 10 μm. **C:** Extrachorion (ex) covering the egg but partially raised in *Ephemera danica* (CPD) (c = chorion), scale bar = 5 μm. **D:** Extrachorion (ex) partially covering the egg in *Caenis pusilla* (CPD) (c = chorion, mp = micropyle, pc = polar cap), scale bar = 10 μm.

Finally, we conclude that the air-drying of eggs following treatment with HMDS or TMS can be considered a good alternative technique to critical point drying, since it is faster and cheaper. Even so, to standardize the procedures established in the SEM study of Ephemeroptera and Plecoptera eggs, we propose that critical point drying technique should be used for the first morphological descriptions, while air-drying after HMDS treatment can be used for confirmation and second descriptions. Because of the problems observed in eggs with a thin or structurally simple chorion, we advise against the use of TMS air-drying until our knowledge of egg radial complexity in these orders of insects is complete.

ACKNOWLEDGMENTS

We thank Mrs. María García, technical assistant director of the microscopy service of the Murcia University, for help with the critical point drying. The English manuscript was revised by Philip Thomas.

REFERENCES

Bengtsson S. 1913. Undersöknigar öfver äggen hos Ephemeriderna. Entomol Tidskr 34:271–320.

Botes L, Price B, Waldron M, Pitcher GC. 2002. A simple and rapid scanning electron microscope preparative technique for delicate "Gymnodinioid" dinoflagellates. Microsc Res Tech. 59:128–130.

Degrange C. 1960. Recherches sur la Reproduction des Éphéméroptères. Trav Lab Hydrobiol Pisc Univ Grenoble. 51:7–193.

Dey S, Basu-Baul TS, Roy B, Dey D. 1989. A new rapid method of air-drying for scanning electron microscopy using tetramethylsilane. J Microsc 156:259–261.

Domínguez E, Cuezzo MG. 2002. Ephemeroptera egg chorion characters: a test of their importance in assessing phylogenetic relationships. J Morphol 253:148–165.

Gaino E, Bongiovanni E. 1992. The egg of *Pseudocentroptilum pennulatum*: a scanning electron microscopy (SEM) study (Ephemeroptera, Baetidae). Boll Soc Entomol Ital 12:195–200.

Gaino E, Bongiovanni E. 1993. Scanning electron microscopy of the eggs of *Palingenia longicauda* (Olivier) (Ephemeroptera: Palingeniidae). Int J Insect Morphol Embriol 22:41–48.

Gaino E, Mazzini M. 1987. Scanning electron microscopy of the egg attachment structures of *Electrogena zebrata* (Ephemeroptera: Heptageniidae). Trans Am Microsc Soc 106:114–119.

Gaino E, Rebora M. 2001. Synthesis and function of the fibrous layer covering the eggs of *Siphlonurus lacustris* (Ephemeroptera, Siphlonuridae). Acta Zool (Stockh) 82:41–48.

Gaino E, Rebora M. 2005. Egg envelopes of *Baetis rhodani* and *Cloeon dipterum* (Ephemeroptera, Baetidae): a comparative analysis between an oviparous and an ovoviviparous species. Acta Zool (Stockh) 86:63–69.

Gaino E, Belfiore C, Mazzini M. 1987. Ootaxonomic investigation of the italian species of the genus *Electrogena* (Ephemeroptera: Heptageniidae). Boll Zool 54:169–175.

Gaino E, Mazzini M, Degrange C, Sowa R. 1989. Etude en microscopie à balayage des oeufs de quelques spèces de *Rhithrogena* Eaton groupe *alpestris* (Ephemeroptera: Heptageniidae). Vie Milleu 39: 219–229.

Goldstein JI, Newbury DE, Echlin P, Joy DC, Roming AD, Jr., Lyman CE, Fiori C, Lifshin E. 1992. Scanning electron microscopy & X-ray microanalysis. A text for biologist, material scientists and geologist. 2nd Ed. New York: Plenum Publishing Press. pp. 810.

Hynes HBN. 1941. The taxonomy and ecology of the nymphs of British Plecoptera with notes on the adults and eggs. Trans R Entomol Soc Lond 91:459–557.

Hynes HBN. 1974. Observations on the adults and eggs of Australian Plecoptera. Aust J Zool (Suppl ser.) 29:37–52.

Isobe Y. 1997. Anchors of Stonefly eggs. In: Landolt P, Sartori M, editors. Ephemeroptera & Plecoptera: biology-ecology-systematics. Fribourg: MTL. pp. 349–361.

Klonowska M. 1997. The use of egg morphology in the taxonomy of some species of the genus *Rhithrogena* (Ephemeroptera: Heptageniidae). In: Landolt P, Sartori M, editors. Ephemeroptera & Plecoptera: biology-ecology-systematics. Fribourg: MTL. pp. 372–381.

Klonowska M, Jazdzewska T. 2003. Scanning electron microscopy study of the eggs of some rare mayfly (Ephemeroptera) species: *Ametropus fragilis*, *Isonychia ignota* and *Neoephemera maxima*. In: Gaino E, editor. Research update on Ephemeroptera & Plecoptera. Perugia (Italy): University of Perugia. pp. 457–462.

Kopelke JP, Müller-Liebenau I. 1981a. Eistrukturen bei Ephemeroptera und deren Bedeutung für die Aufstellung von Artengruppen am Beispiel der europäischen Arten der Gattung *Baetis* Leach, 1815 (Insecta: Baetidae). Teil II: *rhodani-*. vernus- und *fuscatus*-Gruppe. Spixiana. 41:39–54.

Kopelke JP, Müller-Liebenau I. 1981b. Eistrukturen bei Ephemeroptera und deren Bedeutung für die Aufstellung von Artengruppen am Beispiel der europäischen Arten der Gattung *Baetis* Leach, 1815 (Insecta: Baetidae). Teil III: *buceratus-*. atrebatinus-, *niger-*, *gracilis-* und *muticus-* Gruppe. Deutsch entomol Z. 28:1–6.

Kopelke JP, Müller-Liebenau I. 1982. Eistrukturen bei Ephemeroptera und deren Bedeutung für die Aufstellung von Artengruppen am Beispiel der europäischen Arten der Gattung *Baetis* Leach, 1815 (Insecta: Baetidae). Teil I: *alpinus-*. lutheri-, *pavidus-* und *lapponicus-* Gruppe. Gewässer Abwässer 68/69:7–25.

Koss RW. 1968. Morphology and taxonomic use of Ephemeroptera eggs. Ann Entomol Soc Am 61:696–721.

Koss RW, Edmunds GF, Jr. 1974. Ephemeroptera eggs and their contribution to phylogenetic studies of the order. Zool J Linn Soc 55:267–349.

Mazzini M, Gaino E. 1985. Fine structure of the egg shells of *Habrophlebia fusca* (Curtis) and *H. consiglioi Biancheri* (Ephemeroptera: Leptophlebiidae). Int J Insect Morphol Embriol 4:327–334.

Nation JL. 1983. A new method using Hexamethyldisilazane for preparation of soft insect tissues for scanning electron microscopy. Stain Technol 58:347–351.

Rosciszewska E. 1996. Egg capsule structure of the stonefly *Protonemura intricata* (Ris, 1902) (Plecoptera: Nemouridae). Acta Biol Crac Ser Zool 38:41–49.

Smith OR. 1935. The egg and eggs-laying habits of North American mayflies. In: Needham JG, Traver JR, Hsu YC, editors. The biology of mayflies. Ithaca, New York: Comstock Publishing Co., Inc. pp. 67–89.

Soldan T. 1979. The structure and development of the female internal reproductive system in six european species of Ephemeroptera. Acta Entomol Bohemoslov 76:352–365.

Stark BP, Szczytko SW. 1982. Egg morphology and phylogeny in Pteronarcyidae (Plecoptera). Ann Entomol Soc Am 75:519–529.

Stark BP, Szczytko SW. 1988. Egg morphology and phylogeny in Arcynopterygini (Plecoptera: Perlodidae). J Kans Entomol Soc 61: 143–160.

Stark BP, Cox PG, Lentz DL, Hogue AB. 1987. Abnomalities in stonefly eggs (Insecta: Plecoptera). J Kans Entomol Soc 60:588–592.

Studemann D, Landolt P. 1997. Eggs of Ephemerellidae (Ephemeroptera). In: Landolt P, Sartori M, editors. Ephemeroptera & Plecoptera: biology-ecology-systematics. Fribourg: MTL. pp. 362–371.

Studemann D, Tomka I. 1991. European Siphlonuridae (Ephemeroptera): a phylogenetics system for the four genera. In: Alba-Tercedor J, Sánchez-Ortega A, editors. Overview and strategies of Ephemeroptera and Plecoptera. Florida: The Sandhill Crane Press. pp. 103–114.

Tierno de Figueroa JM. 1998. Biología imaginal de los Plecópteros (Insecta: Plecoptera) de Sierra Nevada (Granada, España). Tesis doctoral. Departamento de Biología Animal y Ecología. Universidad de Granada. 310 pp.

Ubero-Pascal N, Soler A, Puig MA. 2001. Contribution to the egg morphology of the Spanish stoneflies (Plecoptera). An overview in the Segura river basin (S.E. of Spain). In: Domínguez E, editor. Trends in research in Ephemeroptera and Plecoptera. New York: Kluwer Academic/Plenum Publishers. pp. 467–478.

Ubero-Pascal N. 2004. Estudio Morfológico del huevo en los Órdenes Ephemeróptera y Plecóptera. Aplicación Taxonómica en la cuenca del Río Segura (S.E. de la Península Ibérica). Tesis doctoral. Departamento de Zoología y Antropología Física. Universidad de Murcia. 580 pp.