



Chorionic fine structure of eggs from some species of *Proboscidoplocia* (Ephemeroptera, Ephemeroidea)

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INTRODUCTION

Recent investigation on Ephemeroptera from Madagascar revealed that mayflies of this area are among the largest in the world (Elouard *et al.*, 1998, 1999) and include even brachypteran species (Ruffieux *et al.*, 1998), thereby making this fauna of particular relevance for phylogenetic studies.

The genus *Proboscidoplocia* Demoulin, 1966 is endemic to Madagascar (Elouard *et al.*, 1999), and since the description of *P. sikorai* (Vayssière, 1895), it has been considered monospecific. Samplings carried out in this Malagasy Region allowed several congeneric species to be described (Elouard & Sartori, 1997; Elouard *et al.*, 1999). Among the characteristics for diagnosis, egg morphology and chorionic structure have given valid support in the new species designation (Sartori *et al.*, 1999). These data emphasise once more the relevance that the chorionic pattern may have in Ephemeroptera, as in many other insect groups, for taxonomic and phylogenetic investigations.

Ephemeroptera ootaxonomy has become an important research field, and papers dealing with the description of new species usually include egg chorionic details in addition to those exhibited by nymphs and adults (Kondratieff & Voshell, 1984; Alba-Tercedor & El Alami, 1999; Thomas *et al.*, 1999; Alba-Tercedor, 2000). Indeed, studies on dipterans and lepidopterans revealed that eggshell synthesis is regulated by gene action (review in Kafatos *et al.*, 1987), a feature consistent with the assumption that the chorionic traits are fixed within a species.

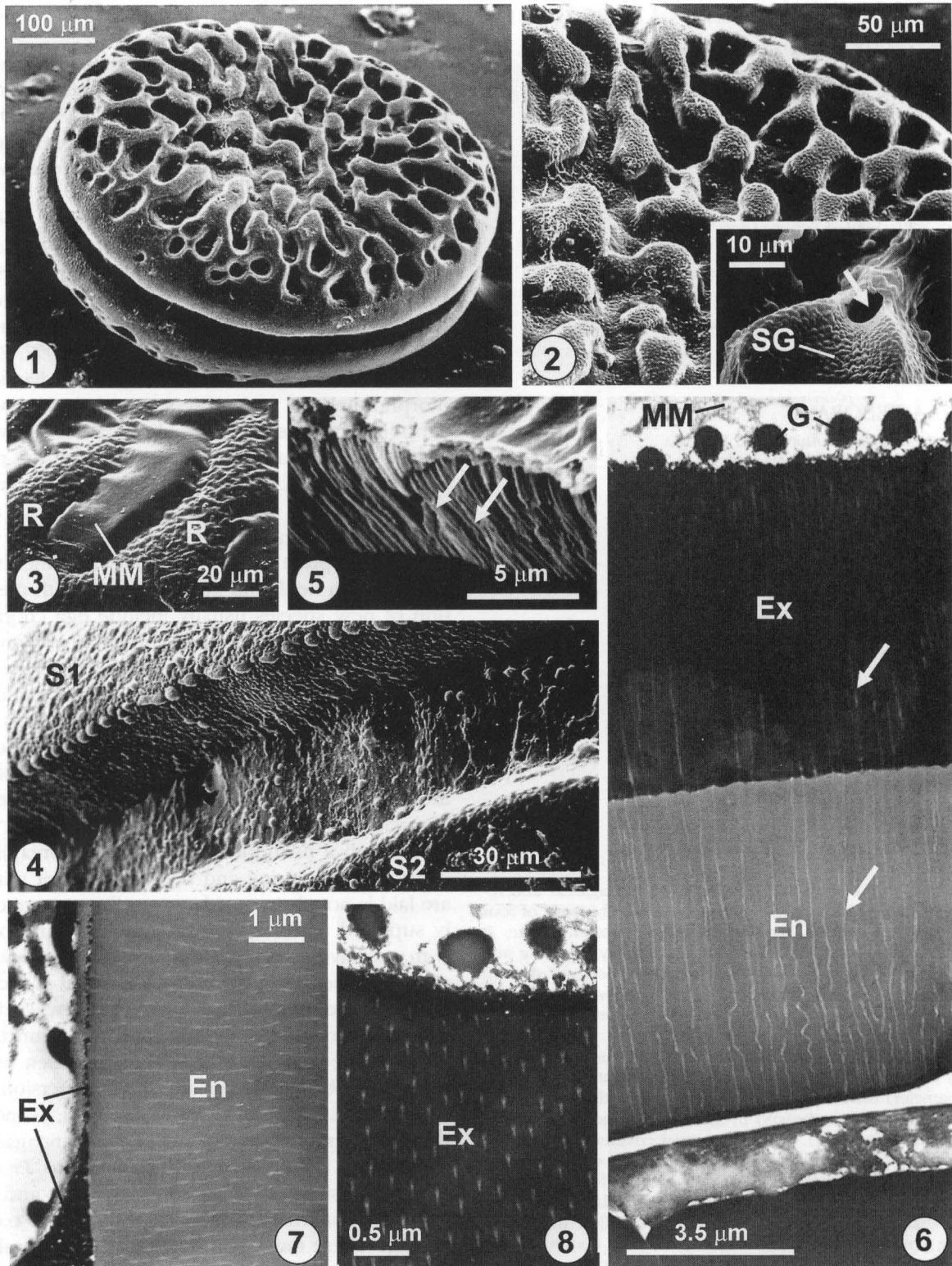
Although a relationship between the organisation of chorionic sculpturing and the environment where eggs are laid is not always evident, mayfly eggs are commonly supplied with attachment structures and/or coated with adhesive layers. On the whole, these devices may provide some advantages for the survival of the species, preventing eggs from being dragged away after their deposition in water. The actual adhesive function attributed to the attachment structures has been repeatedly proved by observing eggs after their immersion in water (Degrange, 1960; Koss & Edmunds, 1974; Gaino & Mazzini, 1987; Provonsha, 1990; Gaino & Flannagan, 1995).

The eggs of the species belonging to *Proboscidoplocia* investigated so far are flattened and show a chorionic pattern varying from decorated to completely smooth. These traits, together with other morphological characteristics of the adults (males and females) are consistent with the assumption that these congeneric species are so closely related as to be considered variants of the same model (Elouard & Sartori, 1997). The aim of the present paper was to study the organisation of the chorion to test whether its fine structure could give insight into this marked relationship.

ABSTRACT

The genus *Proboscidoplocia* is endemic to Madagascar and recent studies revealed that it is not monospecific. The eggs of four newly described species, namely *P. billi*, *P. leplattenerae*, *P. ruffieuxae*, and *P. vayssierei*, were investigated by scanning and transmission electron microscopy. Eggs were flattened and showed two distinct sides joining along their peripheral border. In *P. billi*, a deep groove separated the two sides, whereas only a slight rim was evident in the eggs of the other three species. The chorionic decorations varied from irregular uplifted ribs, as in *P. billi*, to regularly arranged depressions, wider in *P. leplattenerae* (8-10 µm in diameter) than in *P. ruffieuxae* (2-3 µm in diameter). In *P. vayssierei*, the chorion showed irregularly arranged micropores (about 1 µm in diameter). Mucous material covered the egg surface and tended to accumulate in the spaces between ribs or inside the chorionic depressions. The chorion was formed by two layers (endo- and exochorion) that showed a similar organisation of microcanals interposed between adjacent lamellae. In *P. vayssierei*, the presence of tunnels conferred a looser texture on the exochorion. Transmission electron microscopy images showed that both microcanals and tunnels opened on the egg surface. The superficial chorionic pattern was species-specific and the texture of both endo- and exochorion supported the conclusion of a close affinity of these Malagasy species.

KEY WORDS: Malagasy mayflies - Egg envelopes - Chorion - SEM - TEM.



Figs 1-8 - Egg of *Proboscidioplecta billi* under SEM (1-5) and view of the chorionic organisation under TEM (6-8). **1** - Egg *in toto*. Note its sandwich-shaped organisation. **2** - Detail of the chorionic surface showing its decorations of uplifted ribs. In the inset, a zoomed view of the tageniform micropyle constituted by sperm guide (SG) and micropylar opening (arrow). **3** - Mucous material (MM) accumulated in the space between ribs (R). **4** - Deep groove separating the two lateral sides (S1-S2) of the egg. **5** - Mechanically broken chorion showing its organisation in vertically arranged lamellae interspaced by thin canaliculi (arrows). **6** - A section of the chorion showing two superimposed layers: the electron translucent exochorion (Ex) and the electron dense endochorion (En). Note the thin canaliculi (arrows) present in both layers and the electron dense granules (G) located on the outermost surface of the exochorion. Mucous material (MM) with a fibrillar component is interspersed among the granules. **7** - A section of the chorion along the deep groove interposed between the two lateral sides. Note the endochorion (En) and the thin exochorion (Ex). **8** - Pierced organisation of the exochorion (Ex) due to the canaliculi.

MATERIALS AND METHODS

The specimens examined belong to the collection of Elouard and Sartori. Eggs were dissected from the abdomen of the adults of *Proboscidoploecia billi* Elouard & Sartori, 1997 (collected in Manampatrana Basin, a tributary of the Sahavatoiy river, Andringitra Camp 3, 1210 m a.s.l., 22.11.1993), *P. vayssierei* Elouard & Sartori, 1997 (collected in Manampatrana Basin, Andringitra Camp 2, 1400 m a.s.l., 21.11.1993), *P. ruffieuxae* Elouard & Sartori, 1997 (collected in Rianila Basin, Ambodiriana river, PK 231, 30 m a.s.l., 4.11.1994) and *P. leplattenierae* Elouard & Sartori, 1997 (collected in Sahankazo basin, Sakaramy river, Ambre 2, 275 m a.s.l., 5.4.1994). Data on distribution and ecology of these species are reported in Elouard & Sartori (1997) and in Sartori *et al.* (1999).

Since no fresh material was available for ultrastructural investigations, we utilised specimens fixed in alcohol. For scanning electron microscopy (SEM) analysis, eggs were hydrated (using a decreasing graded ethanol series up to water) and left overnight in a small container where some drops of glycerol were added to water in order to remove or reduce the thickness of the mucous material adhering to the egg surface. Thereafter, selected specimens were dehydrated in graded ethanol series, critical point dried using a CO₂ Pabisch CPD apparatus, mounted on stubs with silver conducting paint, coated with gold palladium in a Balzers Union Evaporator, and observed with a Philips EM 515 SEM.

For transmission electron microscopy (TEM) analysis, eggs were first hydrated and then immersed in Karnovsky's medium (Karnovsky, 1965) in cacodylate buffer, pH 7.2, repeatedly rinsed in the same buffer and postfixed for 1 h in osmium tetroxide (1% in cacodylate buffer). Eggs were then rinsed in cacodylate buffer, dehydrated in graded ethanol series, and embedded in Epon-Araldite mixture resin. Thin sections, cut on a Reichert ultramicrotome, were collected on formvar-coated copper grids, stained with uranyl acetate and lead citrate, and observed with a Philips EM 400 TEM and a Philips EM 208 TEM.

RESULTS

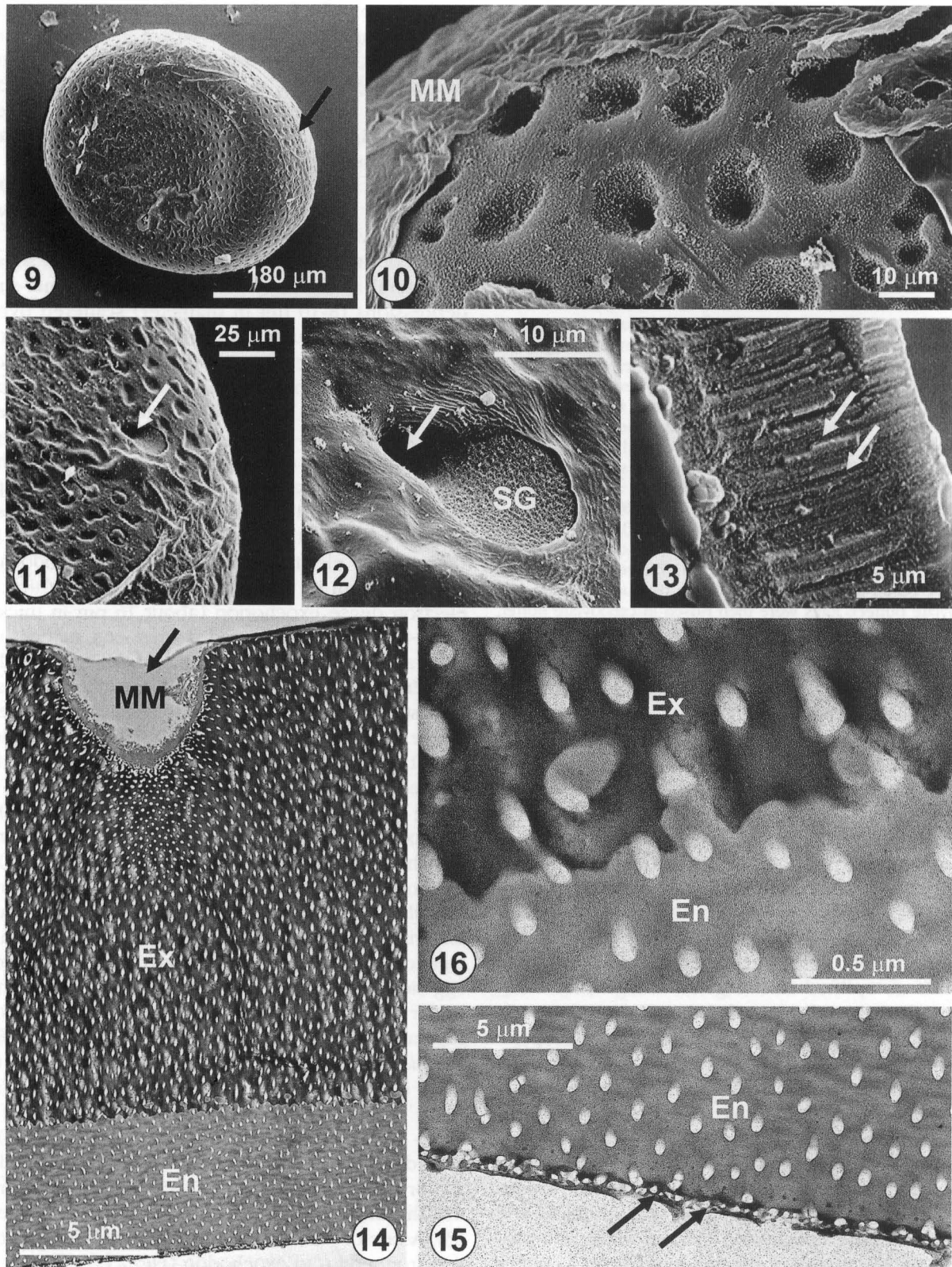
The eggs of the species of *Proboscidoploecia* have a fairly uncommon morphology since they seem to be formed by two distinct sides joining along their peripheral border, and in those of *P. billi* the two lateral sides are separated by a deep groove (Fig. 1). Eggs of *P. billi* measure about 500 µm in diameter and have the same decoration as uplifted ribs (Fig. 2). A tageniform micropyle is evident at the top of a rib and shows a sperm guide, with a granular texture, leading to the micropylar opening (Fig. 2, inset). Ribs are irregularly arranged and the space in between may contain mucous material (Fig. 3). Along the deep groove, the chorion has a simpler organisation (Fig. 4), and in cross section consists of a sequence of tightly packed laminae (Fig. 5). Under TEM, it proves to derive from two superimposed layers: the electron translucent endochorion (7.2 µm thick) and the electron dense exochorion (16 µm thick, as maximum value), which shows aligned granules on its outermost surface and mucous material having a fibrillar component (Fig. 6). The electron dense granules correspond to the irregular chorionic surface evident under SEM (Fig. 4). Apart from the different electron density, both layers show a similar pattern of thin canaliculi corresponding to the intervals between adjacent lamellae (Fig. 6). The endochorion has a uniform thickness,

while the exochorion has a different thickness according to its uplifting into ribs where it reaches its maximum value. Along the deep groove interposed between the two lateral sides, the exochorion is so thin that the eggshell in this region is mainly formed by the endochorion (Fig. 7). In some sections, the thin canaliculi appear as small piercings (Fig. 8).

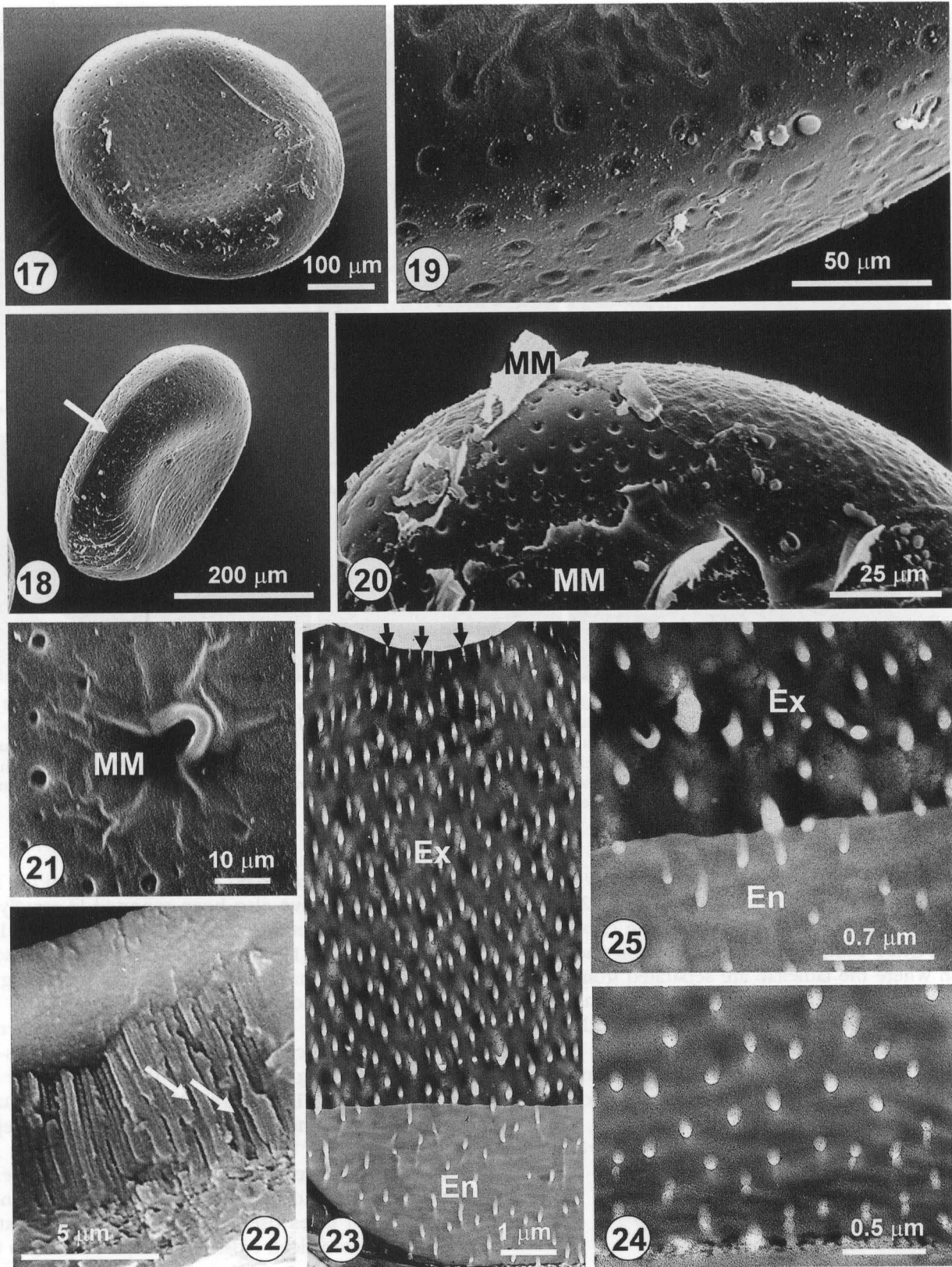
The eggs of *P. leplattenierae* (Fig. 9) measure 400 µm along their maximum diameter, but do not show the deep groove interposed between the two lateral sides typical of the eggs of *P. billi*. Only a slight rim separates the lateral sides. The chorion presents funnel-shaped depressions (8-10 µm in diameter) scattered over the surface, where mucous material accumulates (Fig. 10). This pattern makes the *P. leplattenierae* chorionic surface more uniform than that of *P. billi*. A tageniform micropyle (Fig. 11) is evident along one of the egg sides and consists of a sperm guide with a homogeneous texture and a micropylar opening (Fig. 12). The organisation of the chorion is similar to that of the previous species (Fig. 13). Transmission electron microscopy analysis confirms the basic organisation illustrated for *P. billi*: a more electron translucent and thin endochorion (about 4.5 µm in thickness; Fig. 14) and a thick more electron dense exochorion (about 16 µm in thickness; Fig. 14). The funnel-shaped depressions, evident under SEM, appear as sockets containing fibrillar material (Fig. 14). Endo- and exochorion show a similar organisation consisting in uniformly pierced layers. A more electron dense line is present at the base of the endochorion (Fig. 15), and the transition from endo- to exochorion is marked by a different electron density (Fig. 16).

The eggs of *P. ruffieuxae* measure about 300-400 µm in maximum diameter (Fig. 17). Like in *P. leplattenierae*, a slight rim separates the two lateral sides (Fig. 18). The chorionic depressions are small (2-3 µm in diameter) and show fairly regular distribution (Fig. 19). Mucous material adheres to both egg surface (Fig. 20) and the tageniform micropyle located at the periphery of one egg side (Fig. 21). Mechanically broken eggs examined under SEM showed the typical sequence of lamellae separated by thin canaliculi (Fig. 22). Like in *P. billi* and *P. leplattenierae*, the endochorion (about 3 µm in thickness) is less electron dense than the exochorion (about 9 µm in thickness) (Fig. 23). The organisation of both endo- and exochorion is almost the same (Fig. 23), and according to the section surface, they show round (Fig. 24) or oblong piercings (Fig. 25). Some images reveal that the small piercings open on to the egg surface (Fig. 23). A line of different electron density marks the transition from endo- to exochorion (Fig. 25).

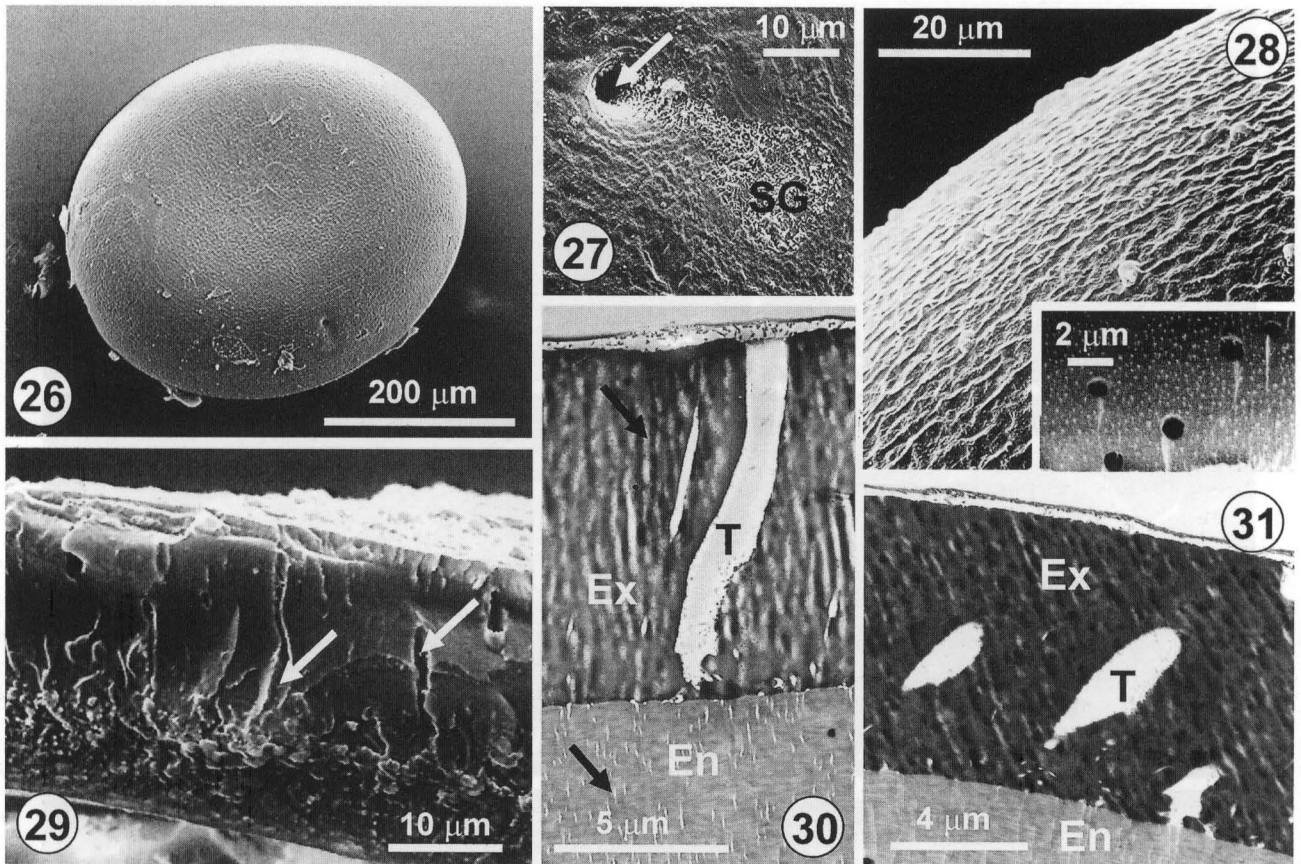
The eggs of *P. vayssierei* measure about 300-350 µm along their maximum diameter (Fig. 26). They are wrapped with adhesive material that is absent in the region of the micropyle, thereby allowing the sperm guide and the micropylar opening to be seen (Fig. 27). On the rest of the egg surface, the mucous envelope forms wrinkles (Fig. 28). When mucus is removed from



Figs 9-16 - Egg of *P. leplattentierae* under SEM (9-13) and view of the chorionic organisation under TEM (14-16). **9** - Egg *in toto*. Note the slight rim (arrow) separating the two lateral sides. **10** - Detail of the egg surface showing the chorion with funnel-shaped depressions. MM, mucous material. **11** - Tageniform micropyle (arrow) on the chorionic surface. **12** - Detail of the micropyle with its sperm guide (SG) and micropylar opening (arrow). **13** - Mechanically broken chorion showing its lamellar pattern with thin canaliculi (arrows) between adjacent lamellae. **14** - A section of uniformly pierced chorion composed of an electron translucent endochorion (En) and an electron dense exochorion (Ex). Note the apical pocket (arrow) containing mucous material (MM). **15** - Detail of the endochorion (En) showing its basal more electron dense organisation (arrows). **16** - A section of chorion at the transition from endo- (En) to exochorion (Ex).



Figs 17-25 - Egg of *P. ruffieuxae* under SEM (17-22) and view of the chorionic organisation under TEM (23-25). **17** - Egg *in toto*. **18** - Lateral view of the egg showing the slight rim (arrow) separating the two lateral sides. **19** - Small piercings regularly distributed on the chorionic surface. **20** - Mucous material (MM) adhering to the egg surface. **21** - Mucous material (MM) adhering to the micropyle. **22** - Mechanically broken chorion showing its lamellar organisation giving rise to thin canaliculi (arrows) interposed between lamellae. **23** - A section of uniformly pierced chorion composed of an electron translucent endochorion (En) and an electron dense exochorion (Ex). Note the thin canaliculi that open on the egg surface (arrows). **24** - Endochorion with round piercings. **25** - A clear cut between endo- (En) and esochorion (Ex). In this latter, the piercings tend to be oblong.



Figs 26-31 - Egg of *P. vayssierei* under SEM (26-29) and view of the chorionic organisation under TEM (30-31). **26** - Egg *in toto*. **27** - Detail of the chorionic surface showing the micropyle with its sperm guide (SG) and micropylar opening (arrow). **28** - The adhesive layer forms wrinkles on the chorionic surface. In the inset, a portion of the egg surface showing the chorionic micropores. **29** - Mechanically broken chorion showing the arrangement of the tunnels (arrows) that open on the outermost egg surface. **30** - A section of the chorion showing thin canaliculi (arrows) in both endo- (En) and exochorion (Ex). In the latter, a tunnel (T) opens outside. **31** - The presence of tunnels (T) is responsible for the loose texture of the exochorion (Ex). En, endochorion.

the egg surface, the chorion shows irregularly arranged micropores (about 1 μm in diameter) (Fig. 28, inset). Mechanically broken eggs show that the chorion consists of a more compact basal endochorion and an exochorion where the lamellae are separated from one another by long tunnels extending from its base to the egg surface (Fig. 29). Under TEM, the endochorion (about 7 μm in thickness) and the exochorion (about 11 μm in thickness) present a similar texture consisting in thin canaliculi (Fig. 30) with additional and differently oriented tunnels in the exochorion (Figs 30, 31). The micropores scattered over the chorion represent the opening of the tunnels on the egg surface.

DISCUSSION

Polymitarcyidae, to which the genus *Proboscidoplocia* belongs (McCafferty, 1991), encompasses, among others, the genus *Euthyplocia*, whose eggs display two distinct 'sides' (Koss & Edmunds, 1974), and also *Mesoplocia* (Sartori M., unpubl. data). This feature is particularly relevant in *P. billi* where the egg shows a sand-

wich-shaped morphology. Eggs differing from the typical oval shape have also been reported in mayfly belonging to Palingeniidae (Gaino & Bongiovanni, 1993; Sartori & Elouard, 1999), as well as in Plecoptera, where this morphology has been related to the increasing egg packing in the oviducts (Stark & Szczytko, 1988).

Under SEM, the chorionic pattern of the congeneric species of *Proboscidoplocia* appears to be species-specific, thus stressing the relevance that ootaxonomy may have in insects (Hinton, 1981; Malzacher, 1982; Mazzini *et al.*, 1984; Gaino *et al.*, 1989; Klonowska-Olejnik, 1997).

Transmission electron microscopy analysis revealed that endo- and exochorion consist of a similar lamellar structure that gives rise to a microcanal-filled texture. Indeed, thin canaliculi cross the bi-layered chorion that, according to the section surface, assumes a pierced configuration. Some images show that the thin canaliculi reach the outermost egg surface. In *P. vayssierei*, in addition to these canaliculi, the chorion is looser owing to the presence of tunnels opening via micropores on the surface. A similar loose texture has been described in some odonates (Degrange, 1971; Sahlén, 1994) and

considered a system for enhancing chorion elasticity (Sahlén, 1994). It seems also acceptable that such an organisation may facilitate oxygen and water exchange, the main problem for eggs laid in water. In some plecopterans, a network of canals has been described as individual components of the aeropylar system (Rościszewska, 1991). The meshwork of canaliculi is a feature common to all the eggs of the examined species of *Proboscidoplocia*. The thickness of the chorion differs from species to species, as does the outermost chorionic pattern. *P. ruffieuxae* has the thinnest chorion whereas *P. vayssierei* can be easily distinguished from the congeneric species owing to the tunnelled exochorion.

The eggs of *Proboscidoplocia* lack attachment structures and are wrapped with a thin coat of mucous material showing a fibrillar component. In *P. billi*, mucous material accumulates among ribs whereas in *P. leplattenierae*, *P. ruffieuxae*, and *P. vayssierei*, the gradual simplification of the chorionic decorations parallels a uniform covering constituted by a thin envelope. *Proboscidoplocia billi* and *P. vayssierei* live in cold streams of lower order (epi- and metarhithral), whereas *P. leplattenierae* and *P. ruffieuxae* live in warmer water (hyporhithral). It is worth noting that eggs of species living in the same environment show a more abundant mucous coat.

The actual function of the mucous material adherent to the egg surface of Ephemeroptera is still controversial. The absence of reproductive accessory glands (Brinck, 1957) confines the secretion of mucous material to the follicular and epithelial cells of the oviduct (Gaino & Mazzini, 1989, 1990). As in many other insect groups laying eggs in water, the main property of this material seems to be adhesive (Koss, 1968; Koss & Edmunds, 1974; Rościszewska, 1996). This assumption is particularly true in Palingeniidae where thick fibrous plaques on both sides of the egg surface originate a sort of stalk (Gaino & Bongiovanni, 1993) anchoring the settled eggs at a certain distance from the muddy substrate (Kosova, 1967). In *Siphonurus lacustris* it has been experimentally proved that, after egg deposition in water, the fibrous layers of tightly entwined filaments enveloping eggs promote marked cohesion of the eggs and their firm adhesion to the substrate (Gaino & Reborá, 2000). Epichorionic coats could enhance the passage of the mature eggs through the oviduct (Gaino & Mazzini, 1989) and protect them during storage in female apparatus (Gaino & Bongiovanni, 1993). Since laid eggs are exposed to environmental aggression, another function of the adhesive layers could be that of preserving eggs from bacterial and fungal attack. This assumption is supported by the finding of antibacterial peptides associated with the egg surface in the mucus, which is secreted by the female reproductive accessory glands and discharged into the common oviduct of the sexually mature medfly *Ceratitis capitata* (Rosetto *et al.*, 1996; Marchini *et al.*, 1997).

In conclusion, egg shape, mucous envelopes and fine chorionic organisation, taken together, may provide

useful indications on both the affinity among species and the physiological and ecological contribution to reproductive success.

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