

# Transmission Electron Microscopic Observations of the Egg Membranes of a South African Heel-walker, *Karoophasma biedouwensis* (Insecta: Mantophasmatodea)

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## Abstract

The ultrastructure of the egg membrane of a South African heel-walker, *Karoophasma biedouwensis* (Mantophasmatodea), was examined using transmission electron microscopy (TEM). The chorion was found to be composed of three layers: an outermost surface layer, an exochorion and an endochorion. Around the anterior pole of the egg, a circular ridge was found on the chorion. In the chorion anterior to the circular ridge, the exochorion was fused with the endochorion into an exo-endochorion. The vitelline membrane was extremely thin, with an electron density higher than those of the chorions. The ultrastructure of the egg membrane system of *K. biedouwensis* was compared with that of another heel-walker, *Mantophasma zephyra*, and those of other orthopteroid groups. A comparative study based on the ultrastructures of the egg membranes suggested that the ultrastructural features of egg membranes may be common in Mantophasmatodea but could not yet designate any orthopteroid groups as candidates of mantophasmatodean relatives (see *Appendix*).

## Introduction

Egg membranes of heel-walkers belonging to a new insect order, Mantophasmatodea, have been examined by scanning electron microscopy (SEM) (Klass *et al.*, 2002; Zompro *et al.*, 2002; Machida and Tojo, 2003; Machida *et al.*, 2004). In a Namibian heel-walker, *Mantophasma zephyra*, a circular ridge of the chorion was found around the anterior pole of the egg (Klass *et al.*, 2002; Zompro *et al.*, 2002). It marks the boundary of a ‘cap structure,’ a region facilitating hatching as the opercula in eggs of phasmids and web-spinners. Hexagonal network patterns of grooves were found on the chorion surface posterior to the circular ridge (Klass *et al.*, 2002; Zompro *et al.*, 2002). These grooves were bridged by delicate trabeculae. In a vertical section, the chorion was composed of three distinct layers: an outermost surface layer formed by vertical columns representing an open network, a middle layer containing aeropyles, and an innermost surface layer that appeared solid (Zompro *et al.*, 2002). Machida and Tojo (2003) and Machida *et al.* (2004) observed the

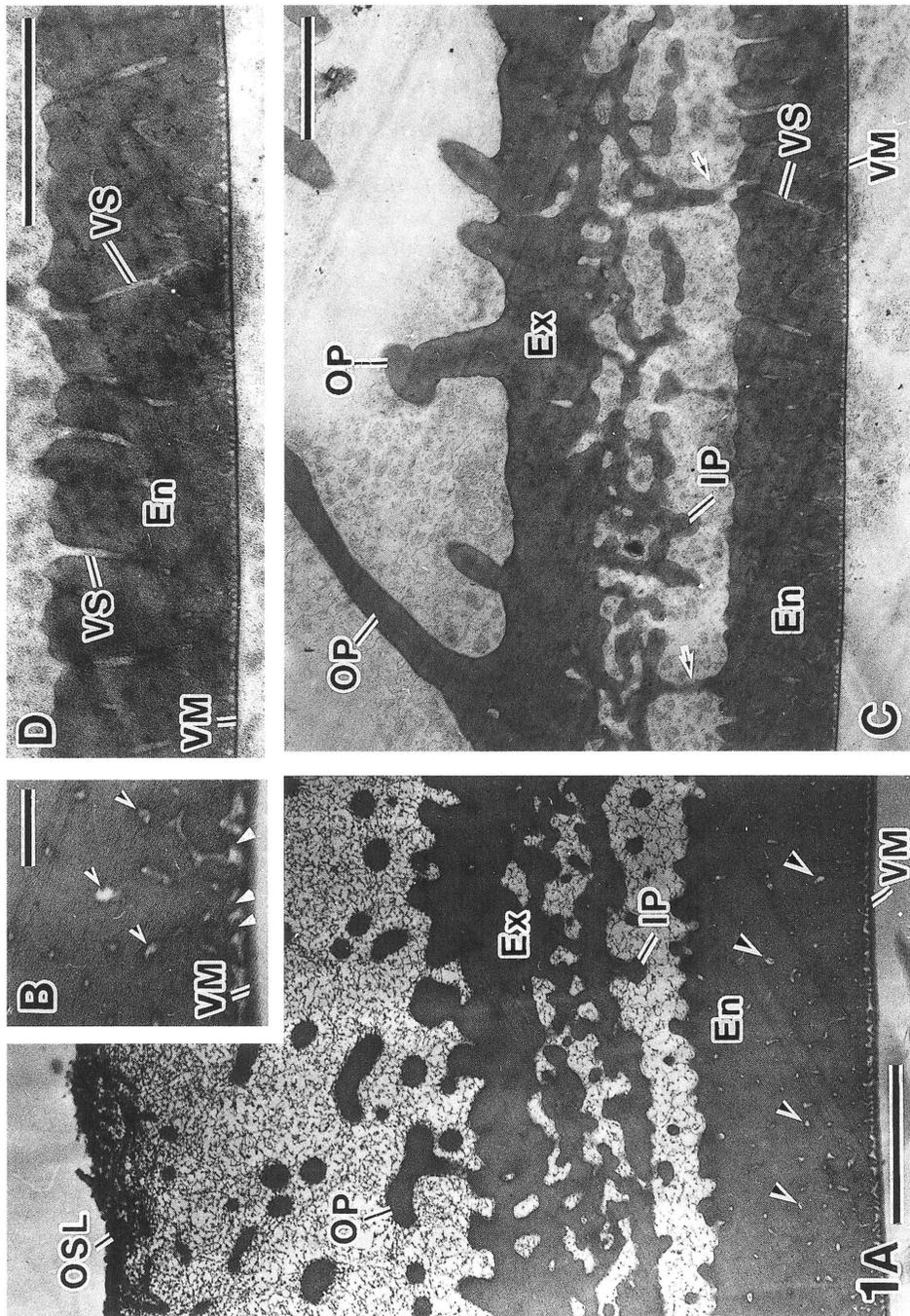


Figure 1

chorion of a South African heel-walker, *Karoophasma biedouwensis*, by SEM and found that it also contained vertical columns and had a faint honeycomb pattern on its outermost surface layer. However, the egg membranes of Mantophasmatodea have never been studied by transmission electron microscopy (TEM).

In the present study, we examined egg membranes of *K. biedouwensis* using TEM and compared the ultrastructural features with those of *M. zephyra* and other orthopteroid groups.

### Materials and Methods

Adult females of *Karoophasma biedouwensis* were collected in September 2002 in Namaqualand (Western Cape Province, South Africa). For TEM, the ovaries containing oocytes and mature eggs were removed from their bodies kept in 70% ethyl alcohol and were fixed with Karnovsky's fixative (2% paraformaldehyde + 2.5% glutaraldehyde) buffered with 0.1 M HCl-sodium cacodylate. The eggs laid by reared females were also fixed with the same fixative. These ovaries and eggs were post-fixed with 1% osmium tetroxide, dehydrated in a graded acetone series, embedded in water-miscible epoxy resin, Quetol 651 (Nisshin EM, Tokyo), or low viscosity Spurr's epoxy resin (Nisshin EM, Tokyo), and cut into ultrathin sections. These sections were double-stained with uranyl acetate and lead citrate and were observed under a transmission electron microscope (JEOL, JEM 1010) at 80 kV.

### Results

The eggs of *Karoophasma biedouwensis* were of an ellipsoid shape with a long diameter of 1.9–2.5 mm and short diameter of 1.0–1.2 mm (cf. Machida and Tojo, 2003; Machida *et al.*, 2004). Structures relevant to micropyles or micropylar plate as seen in eggs of phasmids were not found (cf. Klass *et al.*, 2002; Zompro *et al.*, 2002; Machida *et al.*, 2004). The egg membrane was composed of a three-layered chorion and an extremely thin monolayered vitelline membrane (Fig. 1).

#### Chorion

The chorion was composed of an outermost surface layer (less than 1  $\mu\text{m}$  in thickness), a vertical columnar exochorion (7–11  $\mu\text{m}$  in thickness), and a solid endochorion (2–4  $\mu\text{m}$  in thickness) (Fig. 1). The outermost surface layer was thin and composed of highly electron-dense material (OSL in Fig. 1A) compared to the other two layers of chorion. The outermost surface layer must be a secondary egg membrane, because this layer has been found even in oocytes surrounded by a degenerating follicular epithelium (data not shown).

The exochorion was composed of homogeneously electron-dense material and exhibited a highly complex structure. It included vertical columns in the form of numerous depressions and hollows, numerous long and thick outward projections (2–5  $\mu\text{m}$  in length, OP in Fig. 1), and numerous short and thin inward projections (0.4–2  $\mu\text{m}$  in length, IP in Fig. 1) connected with each other to form a network. There was a wide space between the exochorion and the endochorion, but the inward projections of the exochorion were sometimes interconnected with the endochorion (arrows in Fig. 1C). The spaces formed between vertical columns of the exochorion and a wide space between the exochorion and the endochorion may later become air-filled spaces, but no structures relevant to aeropyles were found.

The endochorion was composed of homogeneously electron-dense material, similar in electron density to that of the exochorion. It had many small protuberances on its outer surface and numerous microprojections on its inner surface (arrows in Fig. 1B). Many small apertures (arrowheads in Fig. 1A, B) were found in the endochorion of immature eggs obtained from ovaries removed from the bodies in 70% ethanol. In a definitive endochorion of the eggs laid by reared females in the laboratory, however, the number of the small apertures was less, but instead there were

Fig. 1 TEM images of the egg membrane of *Karoophasma biedouwensis*. A. Immature egg membrane of the ovarian egg. B. Enlargement of the endochorion (En) in A. Arrowheads indicate numerous small apertures in the endochorion. Note that the vitelline membrane (VM) is in contact with numerous microprojections (white arrowheads) developed from the endochorionic inner surface. C. Definitive egg membrane of a deposited egg. Arrows indicate the inward projections (IP) interconnecting the exochorion (Ex) with the endochorion. D. Enlargement of the endochorion in C. The egg membrane is composed of a three-layered chorion [outermost surface layer (OSL), exochorion and endochorion] and an extremely thin monolayered vitelline membrane. The exochorion has numerous outward long projections (OP) and numerous inward small projections connected with each other. VS: vertical slit of the endochorion. Scales = A, C, D: 2  $\mu\text{m}$ ; B: 500 nm.

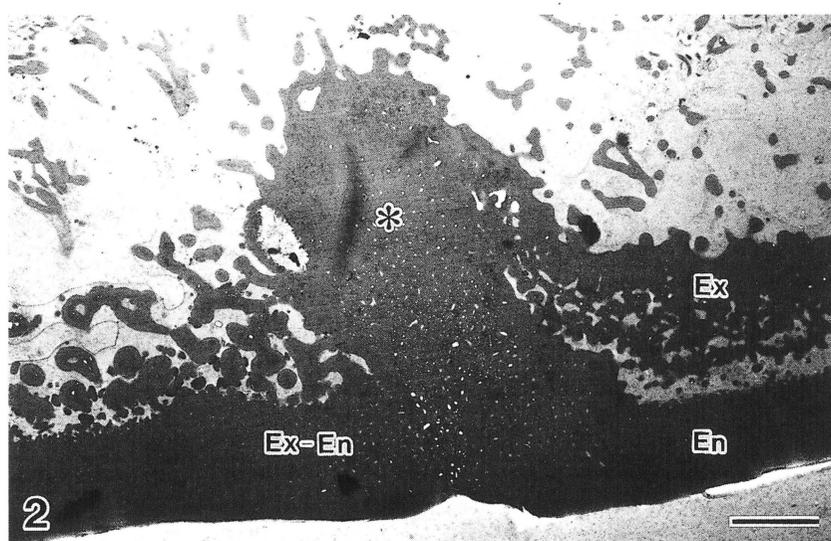


Fig. 2 TEM image showing a sagittal section through the anterior region of a mature egg of *Karoophasma biedouwensis* (anterior to the left). In the circular ridge of the egg, the exochorion (Ex) and endochorion (En) are fused with each other into an exo-endochorion (Ex-En). In the circular ridge, the endochorion becomes remarkably thick and includes numerous small apertures (asterisk). Scale = 4  $\mu\text{m}$ .

numerous vertical fine slits (VS in Fig. 1C, D) penetrating the endochorion.

There were numerous minute particles, which were densely aggregated to form the outermost surface layer and were dispersed through the spaces in the chorion of the immature eggs (Fig. 1A). These particles were also observed on the outward projections of the exochorion. On the other hand, such minute particles were not found in the chorion of the mature eggs (Figs. 1C, 2).

A circular ridge was found in the chorion at the level of *ca.* 15% from the anterior pole of the egg (cf. Machida and Tojo, 2003; Machida *et al.*, 2004). In the chorion anterior to the circular ridge that anteriorly demarcates a 'cap structure,' the exochorion and endochorion were fused with each other into an exo-endochorion, and this area consisted only of two layers: the outermost surface layer and the exo-endochorion (Fig. 2). Thus, the 'cap structure' was structurally continuous with the chorion of main body of the egg. The endochorion in the ridge was characterized by remarkable thickness and numerous apertures (asterisk in Fig. 2).

#### Vitelline membrane

The vitelline membrane was extremely thin (*ca.* 70 nm), with a higher electron density than that of chorion (Fig. 1). Since the vitelline membrane was in contact with numerous microprojections developed from the endochorionic inner surface (white arrowheads in Fig. 1B), there seemed to be numerous small pores between the vitelline membrane and the endochorion.

#### Discussion

##### Ultrastructure of the egg membranes of Mantophasmatodea

The egg membrane system of *Karoophasma biedouwensis* showed a close similarity to that of *Mantophasma zephyra*. The characteristics of egg membranes such as a circular ridge around the anterior pole of the egg, which may be a region facilitating hatching, a thick exochorion containing vertical columns (a network of air-filled spaces) and the lack of a micropylar plate may be common in this insect order. However, we have another interpretation of some structures observed by SEM in *M. zephyra* by Zompro *et al.* (2002). We regard the outermost surface layer, composed of vertical columns, of the chorion in *M. zephyra* as a part of the exochorion, based on the continuity of these two layers. It is likely that the layer with hexagonal network patterns on the chorion surface posterior to the circular ridge is an equivalent of the outermost surface layer of *K. biedouwensis*, which was found to be composed of highly electron-dense

material. Machida *et al.* (2004) revealed by SEM that the outermost surface layer is rough in structure and that in some places it forms a faint honeycomb pattern, of which a hexagon is *ca.* 10  $\mu\text{m}$  in diameter. Zompro *et al.* (2002) regarded the minute air spaces in the middle layer of the chorion (exochorion) of *M. zephyra* as aeropyles. Although these spaces were also observed in the exochorion of *K. biedouwensis*, they were not structurally canals but continuous spaces (cf. Fig. 1). Vertical fine slits in the endochorion of *K. biedouwensis* may be adopted as a candidate for aeropyles (cf. Fig. 1C, D).

Some differences were found between the egg membranes of immature and mature eggs of *K. biedouwensis*. Numerous minute particles were observed in the chorions of immature eggs, while not found in those of mature eggs. It is probable that these particles should have disappeared, being deposited to the outer surface layer and on the surfaces of exochorion and endochorion before the oviposition. The small apertures were numerous observed in the endochorion of immature eggs, but hardly in that of mature eggs, instead there were numerous vertical fine slits penetrating the endochorion (Fig. 1A, B vs Fig. 1C, D). It is likely that the small apertures found in the endochorion of immature eggs are combined together to form the fine slits (probably aeropyles) in the endochorion of mature eggs.

#### *Comparison with egg membrane systems of orthopteroid groups*

Table 1 summarizes the ultrastructural features of egg membranes in Mantophasmatodea and other orthopteroid groups (data for those of Dermaptera and Zoraptera not available). The chorion of Mantophasmatodea was composed of three layers, as in those of caeliferan Orthoptera and Plecoptera. But the outermost surface layer of the chorion of Mantophasmatodea was a part of chorion (a secondary egg membrane), while those of caeliferan Orthoptera and Plecoptera were an extrachorion (a tertiary egg membrane).

The ultrastructures of the exochorion and the endochorion of Mantophasmatodea are similar to those of Phasmatodea: the chorions of both taxa are composed of a sublayered exochorion with a vertical columnar structure and a solid endochorion. These ultrastructural features of the exochorion and endochorion were not found in other orthopteroid groups, such as Orthoptera, Plecoptera, Blattodea, Mantodea and Isoptera (see the column 'Chorion, Ultrastructure' in Table 1). On the other hand, there are remarkable differences between them: vitelline membranes of phasmids are much thicker than those of heel-walkers, and a distinct micropylar plate, operculum and capitulum as shown in phasmid eggs are not observed in the eggs of heel-walkers, as was pointed out by Klass *et al.* (2002) and Zompro *et al.* (2002). The 'cap structure' in Mantophasmatodea eggs seemingly resembles opercula in Phasmatodea and Embioptera eggs, but it is not clear whether the 'cap structure' in eggs of heel-walkers and opercula in eggs of phasmids and webspinners are homologous or not (cf. Machida *et al.*, 2004). Zompro *et al.* (2002) pointed out that the egg of *M. zephyra* is similar to that of Grylloblattodea. Many small apertures were observed in the immature endochorion of *K. biedouwensis* like in *Galloisiana nipponensis* (Matsuzaki *et al.*, 1979), but an essential ultrastructural difference in egg membranes existed among them: the chorion of heel-walkers is subdivided into three layers, whereas that of grylloblattids is monolayered (Matsuzaki *et al.*, 1979). Thus, comparison of ultrastructures of egg membranes does not indicate that Mantophasmatodea are the closest relative of Phasmatodea and/or Grylloblattodea, as proposed by Klass *et al.* (2002).

Dallai *et al.* (2003) showed one of the most parsimonious trees obtained in their cladistic phylogenetic analysis on the basis of seven ultrastructural characteristics of the spermatozoon of *M. zephyra* and related taxa. Their analysis suggested that the closest relationship is between Mantophasmatodea and Mantodea. However, ultrastructural features of egg membranes were considerably different: the chorion of Mantodea consisted of a solid exochorion and laminal endochorion, whereas that of Mantophasmatodea consisted of a vertical columnar exochorion and solid endochorion (Table 1).

The features of the egg membrane system of Mantophasmatodea, such as a circular ridge around the anterior pole of the egg and an extremely fragile vitelline membrane, are unique. A comparative analysis based on the ultrastructure of the egg membrane system could not yet designate any orthopteroid groups as candidates of mantophasmatodean relatives.

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Table 1 Ultrastructures of the egg membranes in Mantophasmatodea and other orthopteroid groups.

|                  | Chorion   |   |  | Vitelline membrane                   | References   |
|------------------|-----------|---|--|--------------------------------------|--|
|                  | Micropyle | Specialized regions facilitating hatching | Ultrastructure   |                                      |  |
| Mantophasmatodea | unknown   | circular ridge (cap structure)            | three-layered; outermost surface layer, exochorion with vertical columns and solid endochorion                     | extremely fragile (70 nm)            | Klass <i>et al.</i> (2002), Zompro <i>et al.</i> (2002), Machida <i>et al.</i> (2004), present study |
| Grylloblattodea  | present   | unknown                                   | monolayered; solid, with numerous apertures  | fragile (0.23 $\mu\text{m}$ )        | Matsuzaki <i>et al.</i> (1979)   |
| Phasmatodea      | present   | operculum + capitulum                     | two-layered; sublayered exochorion with aeropyles and solid endochorion  | thick (0.5–1.0 $\mu\text{m}$ )       | Mazzini <i>et al.</i> (1993)   |
| Mantodea         | present   | hatching line                             | two-layered; solid exochorion and laminal endochorion  | thick (0.7 $\mu\text{m}$ )           | Iwaikawa <i>et al.</i> (1981), Iwaikawa and Ogi (1982)   |
| Blattodea        | present?  | absent                                    | two-layered; thick exochorion with aeropyles and thin laminal endochorion  | fragile (0.2 $\mu\text{m}$ )         | Bellés <i>et al.</i> (1993), Hinton (1981)   |
| Isoptera         | present   | hatching field or absent                  | two-layered; solid exochorion and thin endochorion with aeropyles  | thick (0.6–0.7 $\mu\text{m}$ )       | Grandi (1990), Grandi and Chicca (1999)  |
| Orthoptera       |           |   |  |                                      |  |
| Ensifera         | present   | collar structure or absent                | two-layered; thick solid exochorion and thin endochorion   | various (0.15–0.8 $\mu\text{m}$ )    | Furneaux <i>et al.</i> (1969), Margaritis (1985), Margaritis and Mazzini (1998)                      |
| Caelifera        | present   | absent                                    | three-layered; extrachorion, thin middle layer (exochorion) and thick innermost layer (endochorion) with aeropyles | fragile?                             | Hartley (1961), Margaritis (1985), Margaritis and Mazzini (1998)                                     |
| Plecoptera       | present   | unknown                                   | three-layered; extrachorion, exochorion composed of granular segments and homogeneous endochorion                  | thick (more than 0.5 $\mu\text{m}$ ) | Rósciszewska (1995), Kishimoto (1996)  |
| Embioptera       | unknown   | operculum                                 | unknown concerning subdivision of the chorion  | thick (0.5–0.8 $\mu\text{m}$ )       | Niwa <i>et al.</i> (1993)  |

*Appendix:* Uchifune and Machida (in preparation) recently suggested that the chorion of a Japanese grylloblattid *Galloisiana yuasai* is two-layered and that the vertical slit-like structures (probably aeropyles) develop in the endochorion, as in Mantophasmatodea. If so, the above argument concerning Grylloblattodea might be emended, to suggest a closer affinity between Mantophasmatodea and Grylloblattodea.

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