

# Egg Membranes of *Galloisiana yuasai* Asahina (Insecta: Grylloblattodea)

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## Abstract

The egg membranes of *Galloisiana yuasai* were examined in detail using scanning electron microscopy (SEM) and transmission electron microscopy (TEM), to reconstruct the general features of the egg membrane system of Grylloblattodea, making comparisons with the information available on grylloblattodean egg membranes. The egg membranes of Grylloblattodea are characterized by 1) a two-layered organization of the chorion, 2) numerous aeropyles vertically crossing the endochorion, and 3) a fairly thin vitelline membrane, and these features are shared by Mantophasmatodea, suggesting a closer affinity between Grylloblattodea and Mantophasmatodea.

## Introduction

Grylloblattodea is one of polyneopteran orders, members of which are carnivorous and totally lose their wings, distributed in the northern circumpacific region and central Siberia. Recent analyses on a new polyneopteran Mantophasmatodea, such as comparative embryology (Machida *et al.*, 2004), molecular evolutionary phylogeny (Jarvis and Whiting, 2003), comparative morphology (Klass *et al.*, 2002) and comparative spermatology (Dallai *et al.*, 2005), proposed a possible closer affinity of Mantophasmatodea to Grylloblattodea.

Tsutsumi *et al.* (2004) comparatively discussed the egg membranes of Polyneoptera so as to elucidate the affinity of Mantophasmatodea. However, they failed to designate any orthopteroid groups as candidates of mantophasmatodean relatives. In comparisons, they could utilize, as information on the grylloblattodean egg membranes, only fragmented knowledge from a Japanese grylloblattodean representative *Galloisiana nipponensis*, *i. e.*, brief SEM and TEM observations on the matured oocytes by Matsuzaki *et al.* (1982) and on the deposited eggs by Ando and Nagashima (1982). Our preliminary study on the egg membranes of another Japanese grylloblattodean *Galloisiana yuasai* Asahina, which tested the previous observations on the egg membranes of *Galloisiana nipponensis*, could, however, suggest the possibility of an allied relationship of Mantophasmatodea and Grylloblattodea [see appendix of Tsutsumi *et al.* (2004)].

In this paper, we describe the egg membranes of *Galloisiana yuasai* by SEM and TEM in detail, to reevaluate the egg membrane system of Grylloblattodea and to provide a sound basis for the compariso.. of egg membranes of Mantophasmatodea and Grylloblattodea.

## Materials and Methods

Adult females of *Galloisiana yuasai* were collected at localities of about 1,000 m alt. of Kazawa, Gunma Prefecture and Yunomaru, Nagano Prefecture, Japan, in September, 2003. The ovarioles dissected out of females and eggs laid under rearing conditions were fixed with Karnovsky's fixative (2% paraformaldehyde + 2.5% glutaraldehyde) buffered with 0.1 M HCl-sodium cacodylate.

For TEM, fixed ovarioles and eggs, after immersion in 1% tannic acid for 3 h, were postfixed with 1% osmium tetroxide. They were dehydrated in a graded ethyl alcohol series, embedded in water-miscible epoxy resin, Quetol 651 (Nisshin EM), and processed with an ultramicrotome (RMC MT-XL) into sections 80 nm thick. These sections were stained with uranyl acetate, phosphotungstic acid and lead citrate and observed under a TEM (TOPCON LEM-2000) at

90 kV.

For SEM, the fixed materials, *i. e.*, the oocytes and a part of the follicular epithelium from the fixed ovarioles and the eggs, were dehydrated in a graded ethyl alcohol series and transferred to *t*-butyl alcohol. They were dried in a *t*-butyl freeze drier (VACUUM DEVISE VFD-21S), coated with gold and observed under an SEM (TOPCON SM-300). The remaining halves of the materials used for ultrathin sectioning were immersed in the Maxwell solution [saturated potassium hydroxide absolute methyl alcohol + propylene oxide (2:1) solution; cf. Maxwell (1977)], which resolves the embedding medium, and were observed under an SEM.

## Results

### Eggs

Eggs of *Galloisiana yuasai* are ellipsoidal, with long and short diameters of about 1.6 mm and 0.75 mm, respectively, at laying (Fig. 1). The chorion shows a honeycomb pattern (Fig. 2A; cf. Fig. 5A) and bears numerous, disc-shaped projections about 2  $\mu\text{m}$  tall on its surface (Figs. 2A, C, D, 3B, 4A, B; cf. Figs. 5A, 6). In the anterior egg pole, two to six micropyles about 2.5  $\mu\text{m}$  thick are irregularly arranged in a circle about 150  $\mu\text{m}$  in diameter (Fig. 2A–D), though in some cases they are restricted to one half of the circumference (Fig. 2A). The micropylar canals obliquely penetrate the chorion (Fig. 3B) and open at its inner surface (Fig. 3A).

Egg membranes of *Galloisiana yuasai* consist of the chorion and a vitelline membrane. The chorion is composed of two layers (Fig. 4A, B; cf. Fig. 6), an outer exochorion 0.5–1  $\mu\text{m}$  in thickness with higher electron-density and an inner endochorion of about 7  $\mu\text{m}$  with an electron-density a little less than that of exochorion. Numerous fine aeropyles cross the endochorion (Figs. 3B, 4A; cf. Fig. 6) and are observed to open at the inner surface of the chorion (Fig. 3A): the aeropyles run vertically but are sometimes diverged (Fig. 4C). Cross sections of chorions reveal that the disc-shaped projections are derived only from the exochorion (Fig. 4A, B; cf. Fig. 6). The vitelline membrane is a fairly thin and fragile membrane 0.1–0.2  $\mu\text{m}$  in thickness, being observed just beneath the endochorion (Fig. 4A, C).

### Matured oocyte

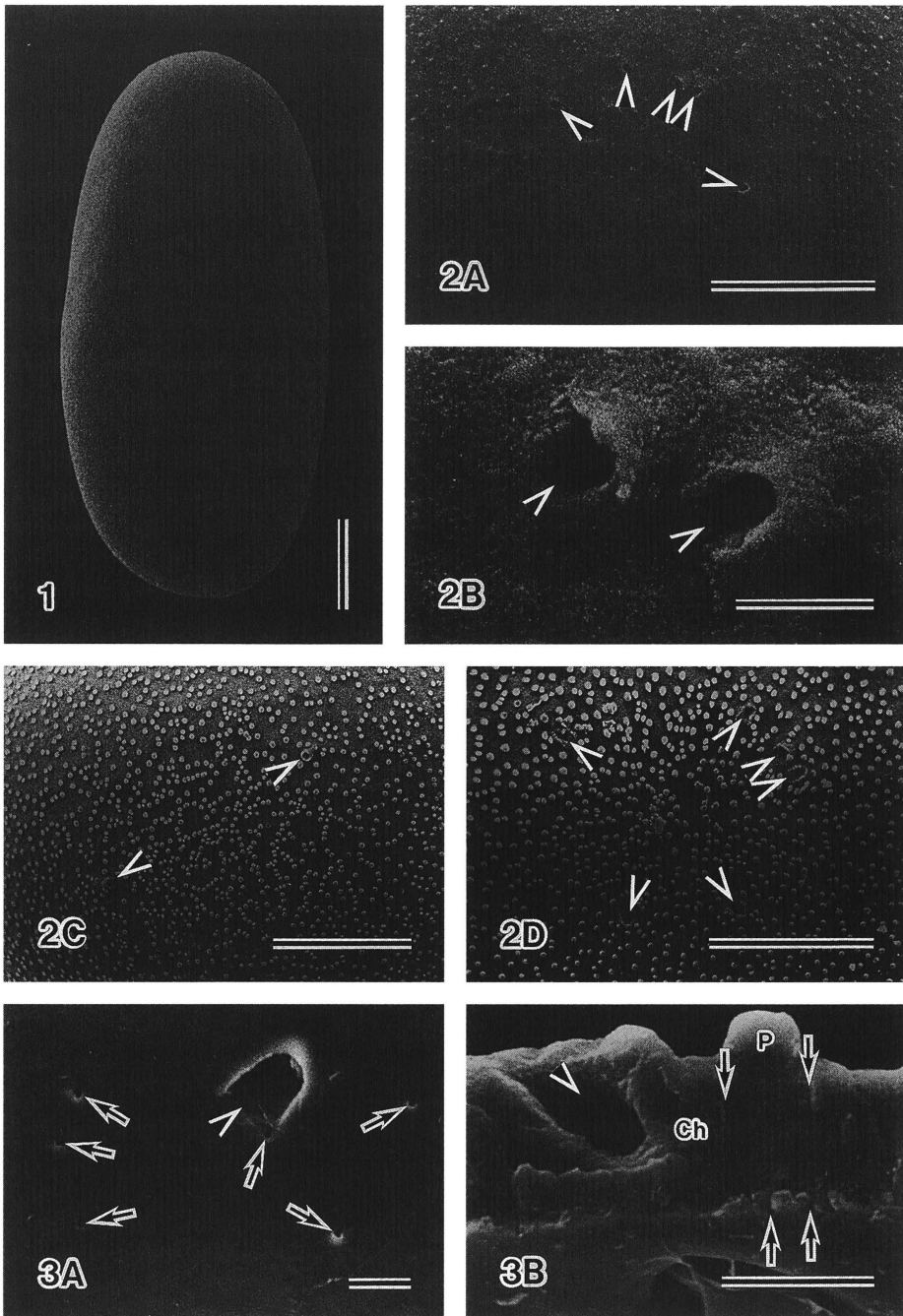
An oocyte taking its position in the proximal-most region of the ovariole or the matured oocyte in *Galloisiana yuasai* has acquired the same characteristics as those of eggs in almost all aspects, *e. g.*, with respect to the general features of the chorion such as the two-layered composition and the possession of aeropyles and sculptures on the exochorion (Figs. 5A, 6). Different from in the egg, however, an additional layer is found to be deposited outside of the exochorion in the matured oocyte. This is a granulated and rough-structured layer a few micron meters in thickness, with higher electron and structural densities towards the chorion (asterisks in Fig. 6). This is demonstrated under SEM as a cortical layer about 1  $\mu\text{m}$  in thickness, covering the surface of the oocyte and subject to peeling off (asterisks in Fig. 7).

The apical surface of the follicular epithelium of the ovariole shows a pattern exactly coinciding with the honeycomb pattern shown in the exochorion (arrowheads in Fig. 5B). Also observed on the surface of the follicular epithelium are depressions probably relevant to the disc-shaped projections on the chorion, although the former was shown to be much fewer in number than the latter (arrows in Fig. 5B).

## Discussion

Matsuzaki *et al.* (1982) examined the egg membranes of Grylloblattodea using the matured oocytes of *Galloisiana nipponensis* as materials. Their observations are in good agreement with our present observations on the egg membranes of *Galloisiana yuasai* in general, except for the interpretation of the organization of the chorion. In the eggs and matured oocytes of *Galloisiana yuasai*, the chorion shows a two-layered organization, composed of the exochorion and endochorion, but Matsuzaki *et al.* (1982) reported that the chorion is one-layered in the matured oocytes of *Galloisiana nipponensis*. However, we may safely deduce that Matsuzaki *et al.* (1982) misread the two-layered chorion to be monolayered, because of almost the same electron-densities of grylloblattodean exo- and endochorion. We can reconstruct the general features of the grylloblattodean egg membrane system as follows: 1) a two-layered chorion composed of an exochorion and endochorion, 2) numerous vertical aeropyles crossing only the endochorion, and 3) a fairly fine, fragile vitelline membrane (cf. Uchifune and Machida, 2005).

Tsutsumi *et al.* (2004) presented detailed comparisons of the egg membranes throughout Polyneoptera. Referring

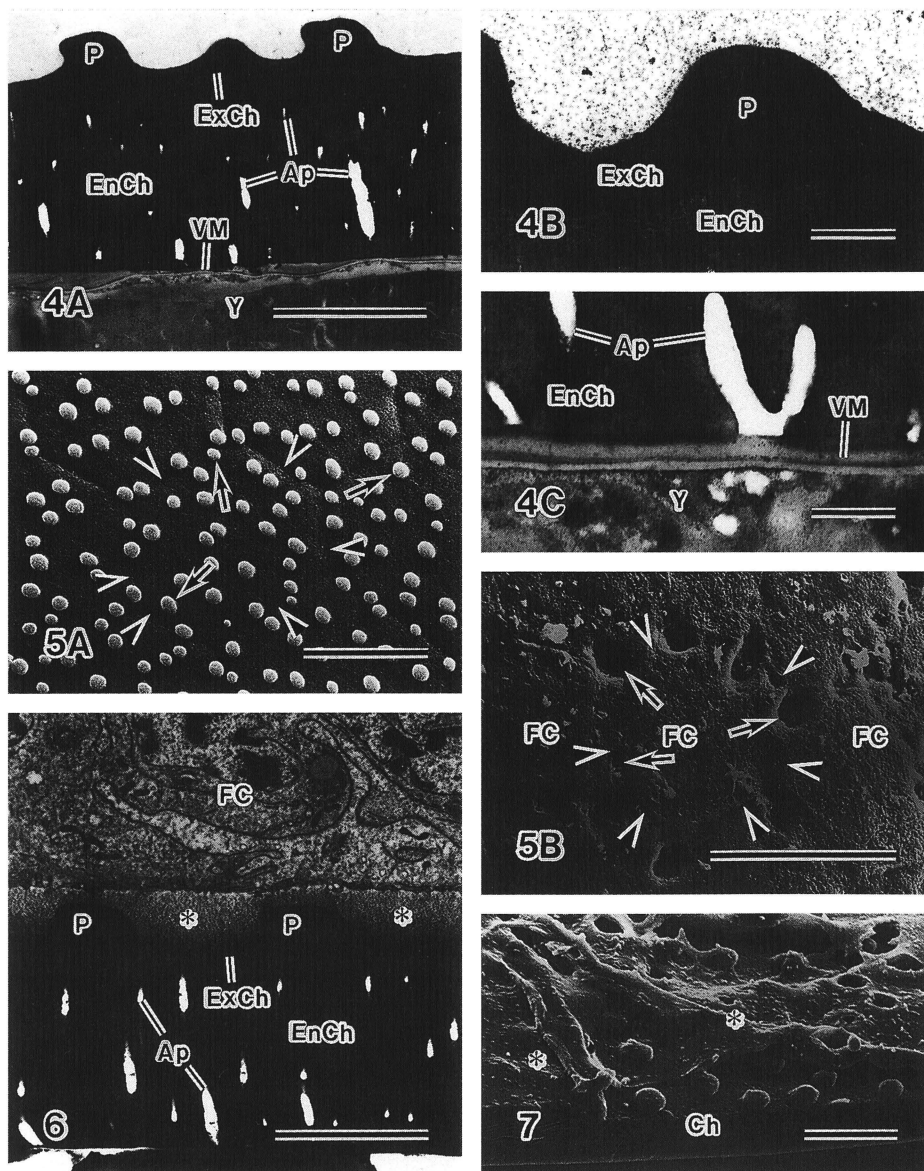


Figs. 1-3 SEMs of egg and chorions of *Galloisiana yuasai*.

Fig. 1 An egg. Anterior egg pole to the top. Scale = 300  $\mu\text{m}$ .

Fig. 2 Micropylar areas. Arrowheads show micropyles. A, C, D. Variation of micropyles in number: five, two and six, respectively. B. Enlargement of the micropyles in A. In A, a honeycomb pattern on the chorion is observed. In A, C and D, disc-shaped projections on the chorion are shown as scattered numerous white spots. Scales = A, C, D: 100  $\mu\text{m}$ ; B: 5  $\mu\text{m}$ .

Fig. 3 Inner surface (A) and fractured facet (B) of the chorion. Arrowheads and arrows respectively show micropyles and aeropyles. Ch: chorion, P: disc-shaped projection. Scales = 5  $\mu\text{m}$ .



Figs. 4-7 Egg membranes and follicular epithelium of *Galloisiana yuasai*.

Fig. 4 TEMs of egg membranes of eggs. A. General features of egg membranes. B, C. Enlargements of outer (B) and inner (C) regions of egg membranes. Scales = A: 5  $\mu\text{m}$ ; B, C: 1  $\mu\text{m}$ .

Fig. 5 SEMs of a matured oocyte (A) and follicular epithelium (B). Arrowheads and arrows respectively show the honeycomb pattern and disc-shaped projections in A and their "printing blocks" in B. Scales = 20  $\mu\text{m}$ .

Fig. 6 A TEM of the egg membranes of a matured oocyte. Asterisks show an electron-dense, granulated layer added to the exochorion. Scale = 5  $\mu\text{m}$ .

Fig. 7 An SEM of the egg membranes of a matured oocyte. The electron-dense, granulated layer added to the exochorion shown by asterisks in Fig. 6 is demonstrated as a covering sheet (asterisks). Scale = 10  $\mu\text{m}$ .

Ap: aeropyle, Ch: chorion, EnCh: endochorion, ExCh: exochorion, FC: follicular cell, P: disc-shaped projection, VM: vitelline membrane, Y: yolk.

to their comparisons, the grylloblattodean egg membranes characterized by the set of features mentioned above prove to be very unique in Polyneoptera, and only Mantophasmatodea shares a set of these features with Grylloblattodea. In this respect, we may suggest a closer affinity between these two polyneopteran orders [cf. Appendix of Tsutsumi *et al.* (2004)], as phylogenetic analyses from various disciplines have already done (see Introduction, cf. Uchifune and Machida, 2005). Other than these features, we may mention the following as shared by Grylloblattodea and Mantophasmatodea. First, the chorions of both orders show a honeycomb pattern on their surface. Second, in a mantophasmatodean *Karooophasma biedouwensis*, an electron-dense, granulated cortical layer was demonstrated to be added to the outer surface of the exochorion, the same as in *Galloisiana yuasai*. However, a similar cortical layer was reported for an isopteran *Reticulitermes lucifugus* (Grandi and Chicca, 1999). Grandi and Chicca (1999) speculated that this layer functions, in *Reticulitermes lucifugus*, as a lubricant which could favor egg advancement along the oviduct as well as helping the reciprocal adhesion of eggs in small heaps. It is probable that the cortical layer in *Galloisiana yuasai* functions as a lubricant for the egg descending along the oviduct, but nothing definite is known.

As discussed, Grylloblattodea and Mantophasmatodea share a common chorionic organization, but we found some differences between them. In Grylloblattodea, the exochorion is rather thinner than the endochorion, whereas in Mantophasmatodea the case is reversed (Tsutsumi *et al.*, 2004). The exochorion of Grylloblattodea is dense in structure with its surface furnished with numerous disc-shaped projections, but that of Mantophasmatodea is composed of numerous columns, showing a spongy appearance. Grylloblattodea and Mantophasmatodea occupy utterly different habitats: the former is a soil-dweller in cold climatic zones, whereas the latter lives in hot and highly arid deserts. These differences in chorion between these two orders are undoubtedly related to the ways they live in extremely different habitats.

In this study, we succeeded in observing the apical surface structure of the follicular epithelium, and found there a similar pattern to the surface structure of the matured oocytes or eggs (Fig. 5A, B). This clearly demonstrates that the follicular cells function as a “printing block” of the chorion. The diameter of the hexagon in the honeycomb pattern in the follicular epithelium is exhibited to be a little shorter than that in the eggs or matured oocytes, and this may be artificially brought about, due to the shrinkage of the follicular epithelium in the course of fixation.

In both *Galloisiana yuasai* and *Galloisiana nipponensis*, micropyles are located around the anterior pole of the egg, on a circular line, but there is a slight difference between them concerning the micropylar arrangement. That is, eight to 10 micropyles are regularly arranged in the latter (Matsuzaki *et al.*, 1982; Ando and Nagashima, 1982), whereas two to six micropyles are irregularly arranged in the former. The number and arrangement of micropyles may be useful in the classification of Grylloblattodea.

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## References

- Ando, H. and T. Nagashima (1982) A preliminary note on the embryogenesis of *Galloisiana nipponensis* (Caudell *et* King). In H. Ando (ed.), *Biology of the Notoptera*, pp. 89–95. Kashiyo-Insatsu Co. Ltd., Nagano.
- Dallai, R., R. Machida, T. Uchifune, P. Lupetti and F. Frati (2005) The sperm structure of Grylloblattodea and their phylogenetic relationships. *Zoomorphology*, **124**, 205–212.
- Grandi, G. and M. Chicca (1999) Oogenesis in supplementary reproductives of *Reticulitermes lucifugus* Rossi (Isoptera Rhinotermitidae): An ultrastructural study. *Invert. Rep. Dev.*, **35**, 65–79.
- Jarvis, K.J. and M.F. Whiting (2003) New insights in grylloblattodean phylogeny. *Entomol. Abh.*, **61**, 146–147.
- Klass, K.-D., O. Zompro, N.P. Kristensen and J. Adis (2002) Mantophasmatodea: A new insect order with extant members in Afrotropics. *Science*, **296**, 1456–1459.
- Machida, R., K. Tojo, T. Tsutsumi, T. Uchifune, K.-D. Klass, M.D. Picker and L. Pretorius (2004) Embryonic development of heel-walkers: Reference to some prerevolutional stages (Insecta: Mantophasmatodea). *Proc. Arthropod. Embryol. Soc. Jpn.*, **39**, 31–39.
- Matsuzaki, M., H. Ando and S.N. Vissher (1982) Fine structure of oocyte and follicular cells during oogenesis in *Galloisiana nipponensis* (Caudell *et* King). In H. Ando (ed.), *Biology of the Notoptera*, pp. 79–87. Kashiyo-Insatsu Co. Ltd., Nagano.
- Maxwell, M.H. (1977) Two rapid and simple methods used for the removal of resins from 1.0  $\mu\text{m}$  thick epoxy sections. *J. Microsc.*, **112**,

253-255.

Tsutsumi, T., R. Machida, K. Tojo, T. Uchifune, K.-D. Klass and M.D. Picker (2004) Transmission electron microscopic observations of the egg membranes of a South African heel-walker, *Karoophasma biedouwensis* (Insecta: Mantophasmatodea). *Proc. Arthropod. Embryol. Soc. Jpn.*, **39**, 23-29.

Uchifune, T. and R. Machida (2005) Embryonic development of *Galloisiana yuasai* Asahina, with special reference to external morphology (Insecta: Grylloblattodea). *J. Morphol.*, **266**, 182-207.