

## Egg structure and outline of embryonic development of the basal mantodean, *Metallyticus splendidus* Westwood, 1835 (Insecta, Mantodea, Metallyticidae)



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### ABSTRACT

The egg structure and outline of the embryonic development of *Metallyticus splendidus* of one of the basal Mantodea representatives, Metallyticidae, were described in the present study. The results obtained were compared with those from the previous studies, to reconstruct and discuss the groundplan of Mantodea and Dictyoptera. In *M. splendidus*, the egg is spheroidal, it has a convex ventral side at the center in which numerous micropyles are grouped, and it possesses a conspicuous hatching line in its anterior half. These are the groundplan features of mantodean eggs and the “grouped micropyles in the ventral side of the egg” are regarded as an apomorphic groundplan feature of Dictyoptera. A small circular embryo is formed by a simple concentration of blastoderm cells, which then undergoes embryogenesis of the typical short germ band type. Blastokinesis is of the “non-reversion type” and the embryo keeps its original superficial position and original orientation throughout embryonic development. During the middle stages of development, the embryo undergoes rotation around the egg’s anteroposterior axis. These features are a part of the groundplan of Mantodea. It is uncertain whether sharing of the “non-reversion type” of blastokinesis by Mantodea and blaberoidean Blattodea can be regarded as homology or homoplasy.

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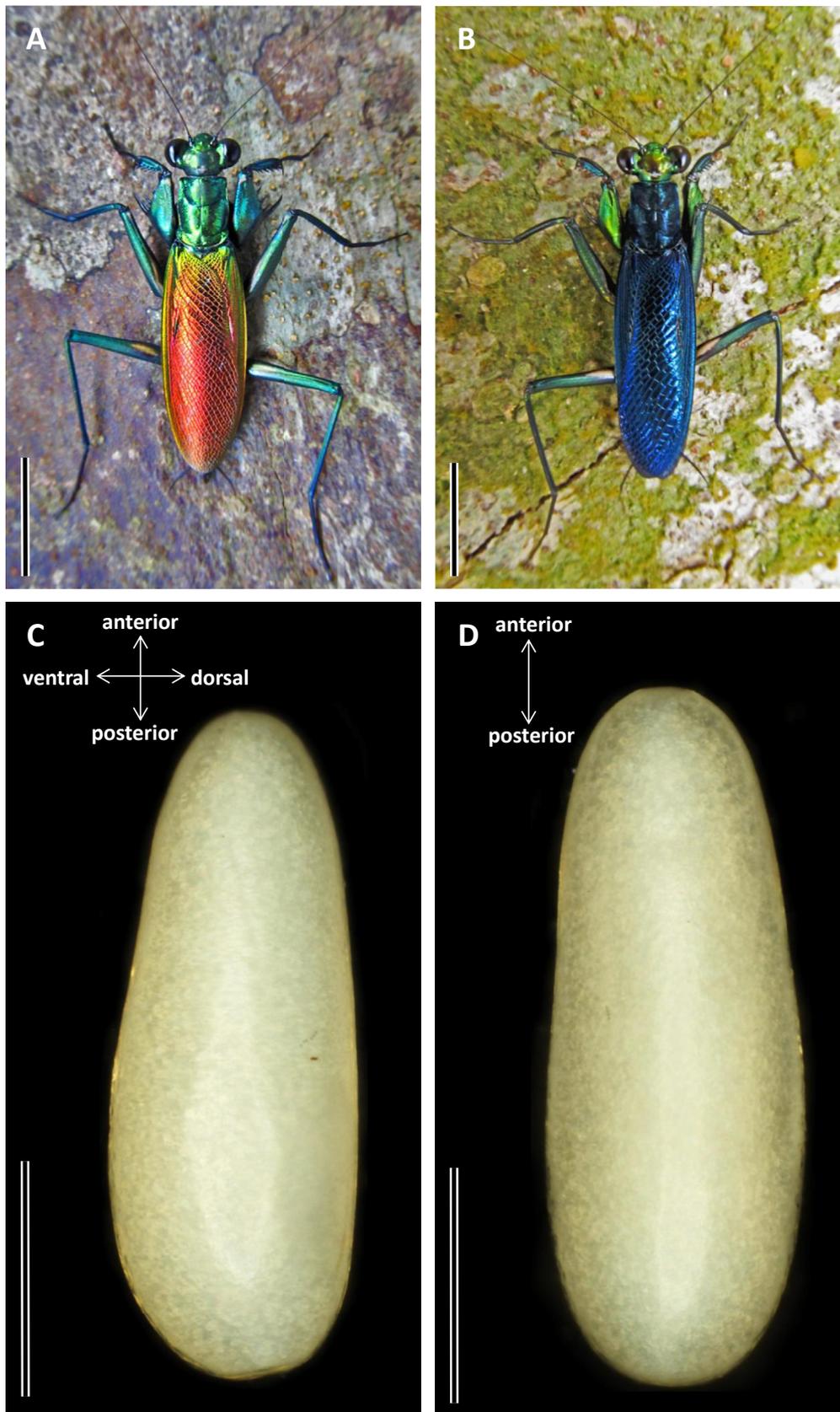
### 1. Introduction

Insects are the most speciose group of animals, accounting for 75% of known animal species. Although their evolution has attracted much attention, studies reconstructing their phylogeny have left several questions unanswered (Trautwein et al., 2012; Kjer et al., 2016). For example, although the phylogeny of Polyneoptera, which comprises 10 lower neopteran orders: Plecoptera, Dermaptera, Embioptera, Phasmatodea, Orthoptera, Zoraptera, Grylloblattodea, Mantophasmatodea, Mantodea, and Blattodea (= “Blattaria” + Isoptera), has been studied in much detail, a

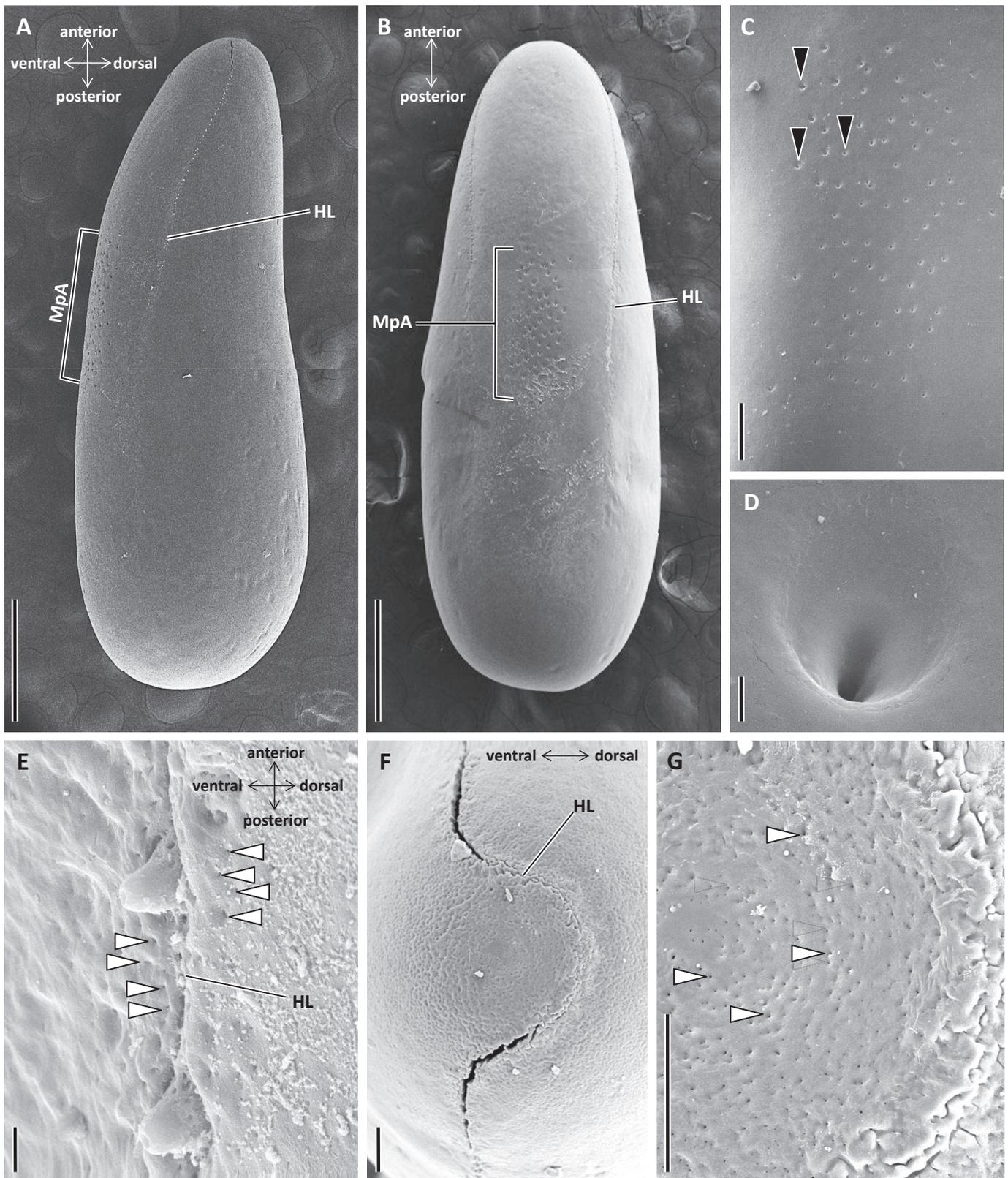
consensus is yet to be reached. Although the status of Polyneoptera has been long argued, recent studies pertaining to comparative morphology, embryology, and molecular phylogenetics strongly suggest that it is most likely monophyletic (e.g., Yoshizawa, 2007, 2011; Ishiwata et al., 2011; Mashimo et al., 2014; Misof et al., 2014), whereas there is no consensus among researchers on the phylogenetic relationships between polyneopteran constituents (Kristensen, 1981, 1991; Grimaldi and Engel, 2005; Terry and Whiting, 2005; Klass, 2009; Beutel et al., 2014). However, the assemblage of Mantodea and Blattodea, i.e., Dictyoptera, has been widely accepted as monophyletic on the basis of various morphological (e.g., Kristensen, 1975, 1981, 1991; Klass, 2003; Beutel and Gorb, 2006; Klass and Meier, 2006; Beutel et al., 2014) and molecular phylogenetic studies (e.g., Maekawa et al., 1999; Wheeler et al., 2001; Kjer, 2004; Terry and Whiting, 2005; Kjer et al., 2006).

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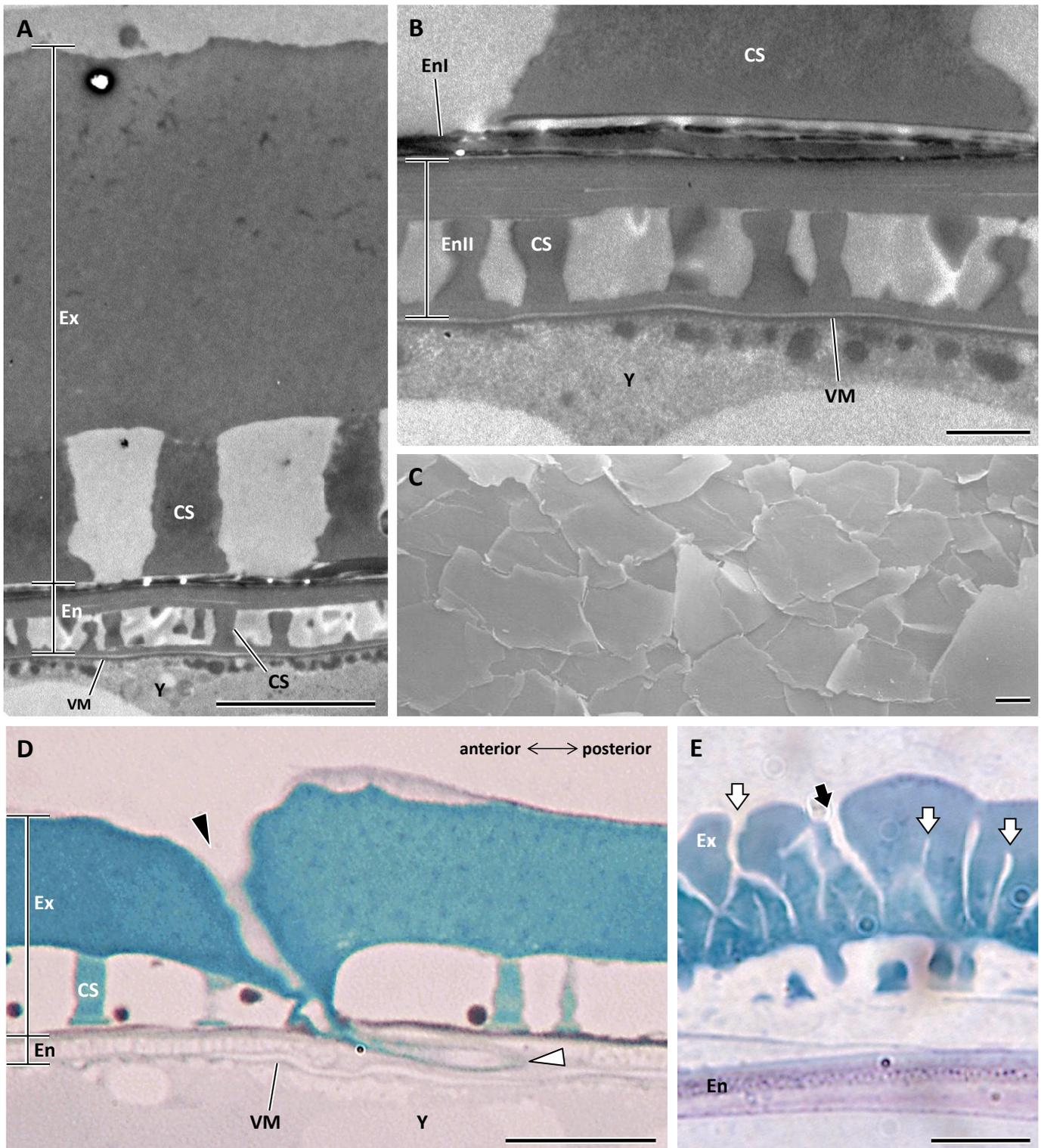
E-mail address: [fukui.makiko.me@ehime-u.ac.jp](mailto:fukui.makiko.me@ehime-u.ac.jp) (M. Fukui).



**Fig. 1.** *Metallyticus splendidus* adults and eggs. (A) Female (B) Male (C) Lateral view of the egg. (D) Ventral view of the egg. Bars = A, B: 1 cm; C, D: 1 mm.



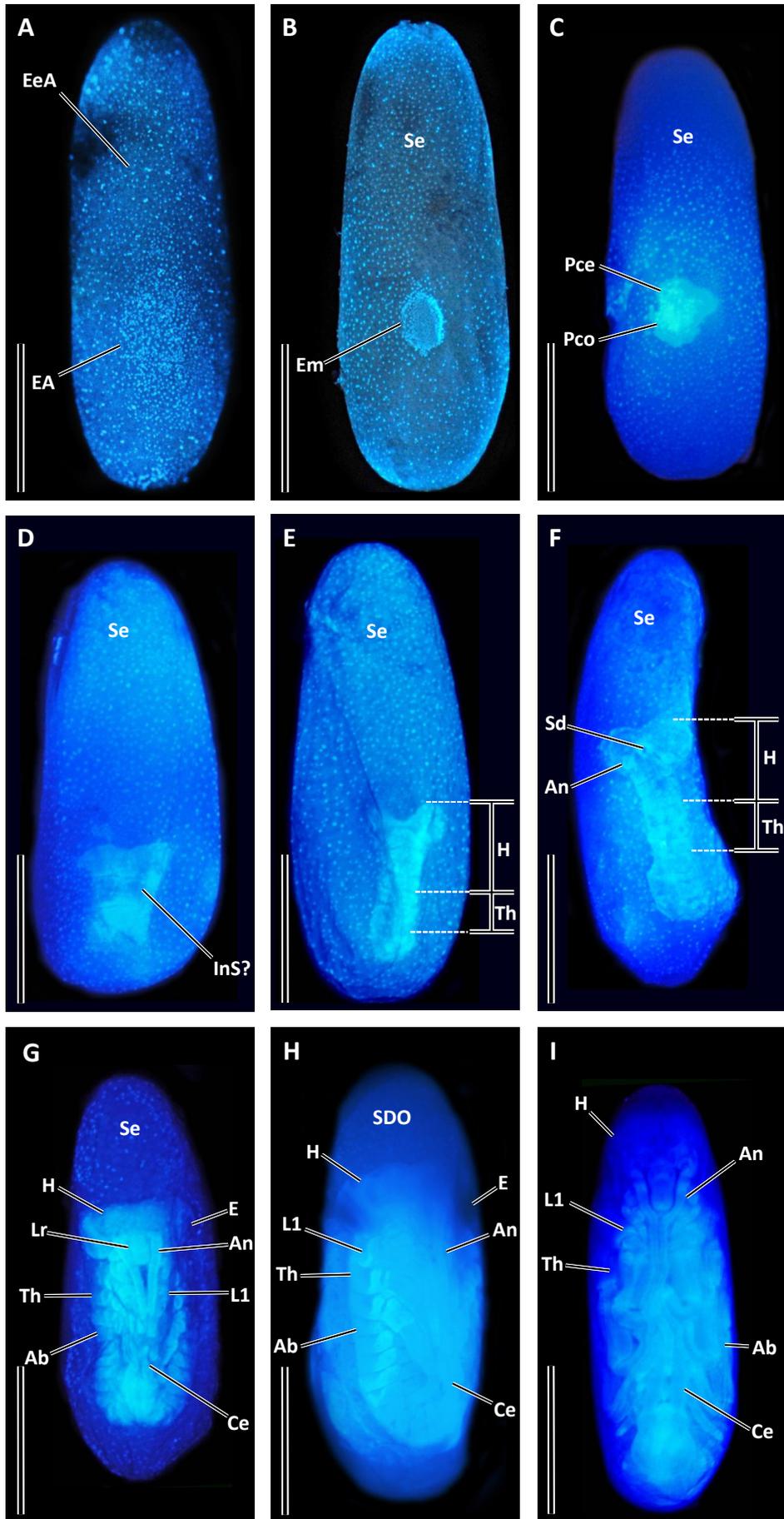
**Fig. 2.** Egg structures of *Metallyticus splendidus*, SEM. (A) Lateral view of egg. (B) Ventral view of egg. (C) Enlarged ventral surface of the egg, showing micropylar area. (D) Close-up of a micropyle. (E) Enlargement of hatching line at the ventral side of the egg. (F) Anterior pole of the egg. (G) Enlargement of F, showing numerous aeropyles. Black and white arrowheads show micropyles and aeropyles, respectively. HL: hatching line, MpA: micropylar area. Bars = A, B: 500  $\mu\text{m}$ ; C: 100  $\mu\text{m}$ ; D, E: 5  $\mu\text{m}$ ; F, G: 50  $\mu\text{m}$ .



**Fig. 3.** Eggshell of *Metallyticus splendidus*. (A) Cross section of eggshell of the ventral side, TEM. (B) Enlarged cross section of eggshell showing the details of the endochorion and vitelline membrane, TEM. (C) Outer surface of the layer I of endochorion, SEM. (D) Sagittal section of a micropyle and micropylar canal. Black and white arrowheads respectively indicate the micropyle and micropylar canal. (E) Sagittal section of eggshell at the anterior pole of the egg, showing the hatching line and aeropylar canals. Black and white arrows indicate the cleft along the hatching line and aeropylar canals, respectively. CS: columnar structure, En: endochorion, EnI: endochorion layer I, EnII: endochorion layer II, Ex: exochorion, VM: vitelline membrane, Y: yolk. Bars = A: 5  $\mu$ m; B: 1  $\mu$ m; C–E: 10  $\mu$ m.

In resolving the phylogenetic issues concerning Polyneoptera, it is important to reconstruct the groundplan of Dictyoptera comprising Mantodea and Blattodea, as well as each polyneopteran order. Comparative embryology is a promising method for reconstructing

the groundplan of a group and discussing its phylogenetic pattern. Recently, reviewing the embryological information covering major blattodean lineages, Fujita and Machida (2017) found that blattodeans undergo two different types of blastokinesis: the “reversion



type,” i.e., blastokinesis with the reversion of the embryo’s anteroposterior and dorsoventral axes, as found in Blattoidea and Isoptera, and the “non-reversion type,” i.e., blastokinesis with the embryo’s axes unchanged, as found in Blaberoidea. Several studies have discussed the embryonic development of the derived family Mantidae of the order Mantodea. These include *Mantis religiosa* (Graber, 1890; Viallanes, 1890, 1891; Giardina, 1897; Cockerell, 1898), *Tenodera* (=Paratenodera) *sinensis* (Hagan, 1917), and *Hierodula crassa* (Görg, 1959). On the basis of these studies, Fujita and Machida (2017) categorized the blastokinesis of Mantodea as the non-reversion type; however, further detailed information on mantodean embryogenesis covering major lineages is required to better understand the groundplan of Mantodea.

Egg structures provide useful information for the reconstruction of insect phylogeny, and the micropyle is one of the significant egg structural features (cf. Hinton, 1981; Mazzini, 1987; Mazzini et al., 1993; Mashimo et al., 2016). Fujita and Machida (2017) suggested that micropyles grouped on the ventral side of the egg to be a groundplan of Blattodea. The micropyles of Mantodea have been reported for a few species of Mantidae (Hagan, 1917; Görg, 1959; Iwaikawa and Ogi, 1982), wherein several micropyles and/or a single large one occur at the anterior pole of the egg. If these descriptions of mantodean micropyles have been correct, Dictyoptera would possess two disparate distribution patterns of micropyles, i.e., both the blattodean and mantodean types.

The inadequate knowledge of the embryology of Mantodea, which is restricted only to a higher group of Mantidae, has been challenging in reconstructing the embryological groundplan of Dictyoptera, and more embryological information is needed on the major mantodean lineages, especially the “basal Mantodea,” i.e., Mantoididae, Chaeteessidae, and Metallyticidae. With this background, we commenced an embryological study of *Metallyticus splendidus* Westwood, 1835, of Metallyticidae. In the present study, we examined its egg structure, outlined its embryonic development, and compared the obtained information with the previous studies on other dictyopterans and polyneopterans, with the aim of discussing the groundplan of Mantodea and Dictyoptera.

## 2. Material and methods

Six females and four males of *M. splendidus* were collected in Rawang, Selangor, Peninsular Malaysia in April 2011. The collected specimens were reared in a laboratory and fed small insects (Fig. 1A, B). We obtained 13 oothecae deposited by females, each of which contained 20–30 eggs. For observing egg structure, the eggs from the newly deposited oothecae were cleaned in 0.1 M phosphate buffered solution (pH 7.2), transferred to Karnovsky’s fixative (2% paraformaldehyde + 2.5% glutaraldehyde in 0.1 M HCl-sodium cacodylate buffer, pH 7.2), perforated with a fine needle to facilitate the penetration of the fixative and fixed overnight. Fixed eggs were rinsed with 0.1 M HCl-sodium cacodylate buffer and observed under an S8 APO Leica stereomicroscope (Heerbrugg, Switzerland). For more detailed observation of their structure, the eggs were postfixed with 1% OsO<sub>4</sub>, dehydrated in a graded ethanol series, dried using a Tousimis Samdri-PVT-3D CO<sub>2</sub> critical-point dryer (Rockville, USA), coated with gold in a JEOL JFC-1100 ion sputter

(Tokyo, Japan), and observed under a TOPCON SM-300 scanning electron microscope (Tokyo, Japan) at an accelerating voltage of 15 kV. Some of the eggs were postfixed with 1% OsO<sub>4</sub> solution, dehydrated in a graded ethanol series, and embedded in an Agar LV epoxy resin (Essex, UK). Semi-thin or ultra-thin sectioning was performed at a thickness of 0.5 μm and 75 nm, respectively, using an RMC MT-XL ultra-thin microtome (Arizona, USA) equipped with a histo-diamond knife. Semi-thin sections were stained using 1% toluidine-pyronine solution or 1% toluidine solution, and observed under an Olympus BH2 biological microscope (Tokyo, Japan). Non-stained ultra-thin sections were observed under a Hitachi HT7700 transmission electron microscope (Tokyo, Japan).

To observe the external features of embryonic development, the exochorion was carefully removed from the eggs and fixed with Karnovsky’s fixative. Fixed materials were stained with DAPI (4’,6-diamidino-2-phenylindole dihydrochloride diluted to about 0.5 μg/ml using distilled water) and observed under a Carl Zeiss SteREO Lumar.V12 fluorescence stereomicroscope (Oberkochen, Germany) or a Leica M165 FC fluorescence stereomicroscope (Heerbrugg, Switzerland) under UV excitation at 360 nm.

## 3. Results

### 3.1. Egg structure

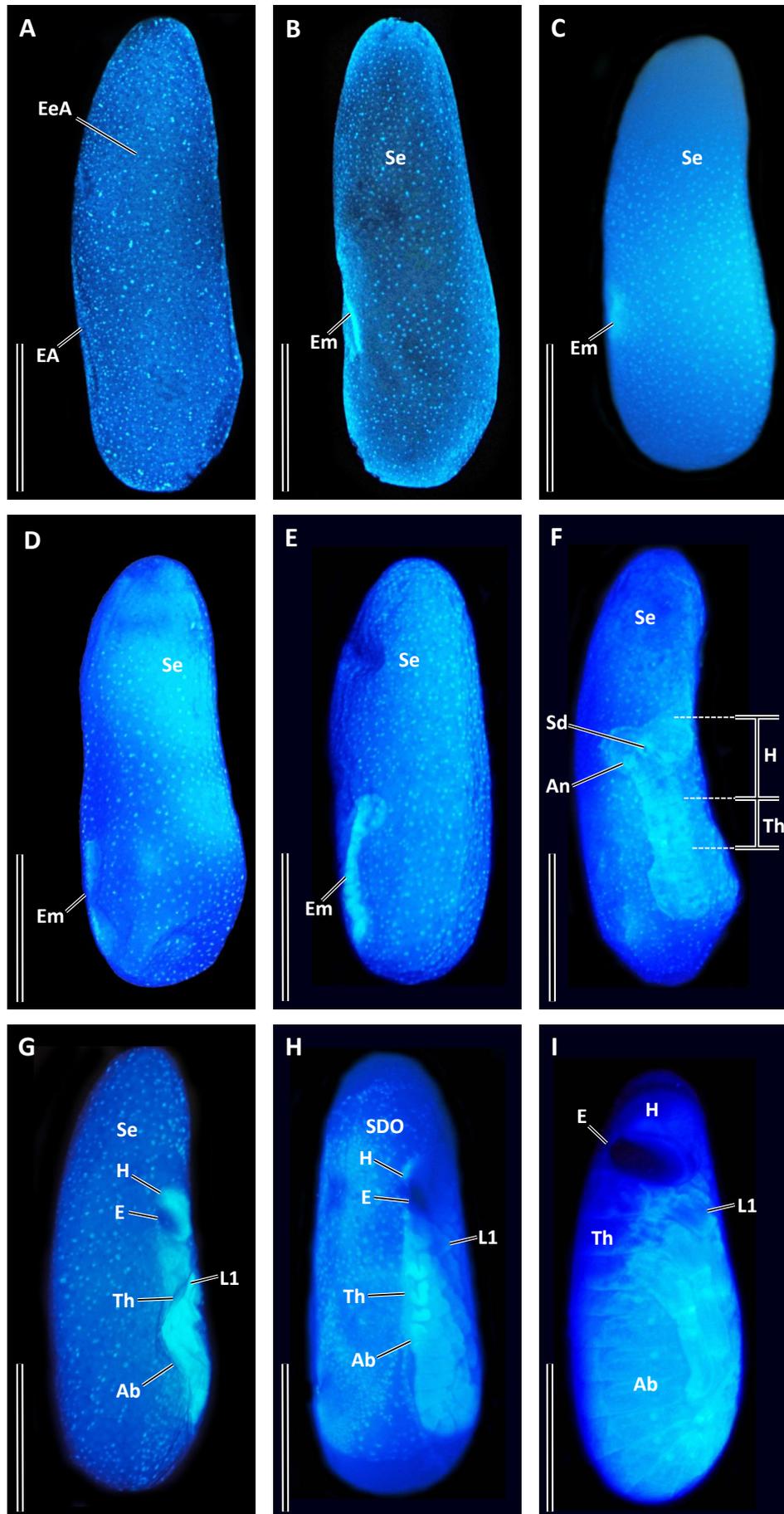
The orientation of insect eggs is defined relative to the embryo position just before hatching (Wheeler, 1893). However, in *M. splendidus*, as in other mantodeans (e.g., Hagan, 1917), the embryos undergo a 180° rotation around the anteroposterior axis of the egg during development. Therefore, we define the ventral side of the egg as the side on which the embryo develops, and the side opposite to it is the dorsal side.

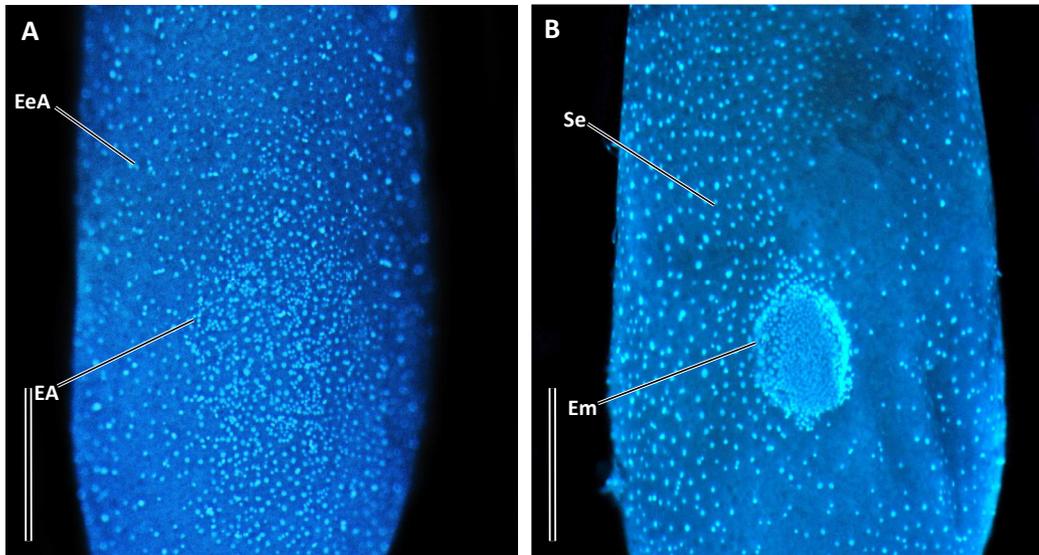
Eggs of *M. splendidus* are yellowish white (Fig. 1C, D) and spheroidal (about 3.2-mm long and 1-mm thick), with a pointed anterior pole, a little convex ventral side, and a straight or slightly concave dorsal side (Figs. 1C and 2A).

A coronal line assumes a long, inverted U-shape at the anterior half of the ventral side of the egg (Fig. 2A, B, E, F). The prolarvae hatch by opening the eggshell along this line, and this may be called the “hatching line.” The hatching line is a deep cleft, as shown in the sagittal section (black arrow in Fig. 3E), and easy to tear. Numerous aeropyles of about 0.5 μm in diameter are seen as deep, branching canals in section (white arrows in Fig. 3E) and are scattered at the anterior pole of the egg and along the hatching line (white arrowheads in Fig. 2E, G). About 80 funnel-shaped micropyles are located in the central area of the ventral side of the egg (Fig. 2A, B, D, black arrowheads in Fig. 2C). Fig. 3D is a sagittal section through a micropyle. The micropylar canal (white arrowhead) penetrates the exo- and endochorion, running obliquely toward the posterior.

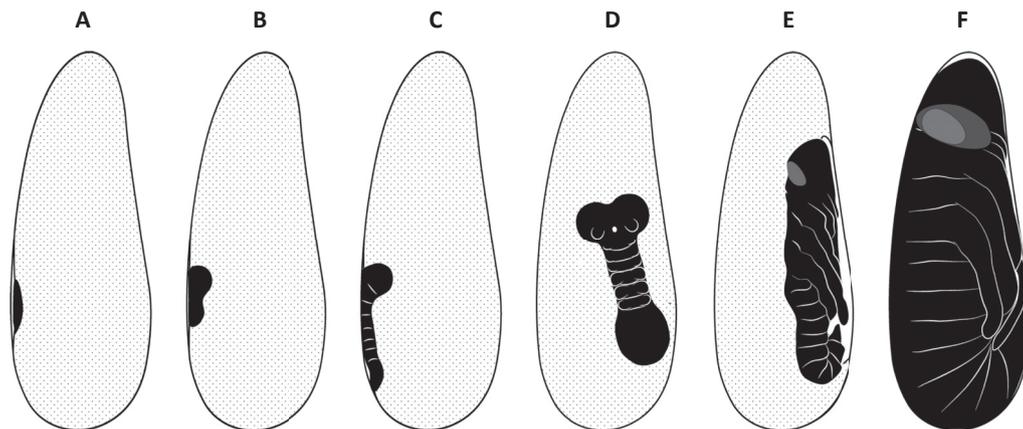
The eggshell comprises three distinct layers: an approximately 15-μm thick exochorion with columnar structures at its bottom, a 2-μm thick endochorion, and an extremely thin vitelline membrane (Fig. 3A, B, D). The endochorion is composed of a thin outer layer (layer I) that has a high electron density and an inner layer (layer II) with numerous, short, columnar structures (Fig. 3B). The layer I shows an appearance of piled laminae (Fig. 3C).

**Fig. 4.** Successive developmental stages of *Metallyticus splendidus*, DAPI staining, fluorescence microscopy, taken at an angle to view the embryos ventrally. (A) Egg at 8 days after oviposition (dao.). The embryonic and extraembryonic areas differentiate. (B) Egg at 11 dao. A small germ disc is newly formed on the ventral side of the egg. (C) Egg at 23 dao. The protocephalon and protocorm differentiate. (D) Egg at 27 dao. The segmentation begins. (E) Egg at 28 dao. The segmentation occurs in the cephalic and anterior thoracic regions. (F) Egg at 30 dao. The segmentation further proceeds, and the segmental appendages develop. (G) Egg at 45 dao. The segmentation is complete, the ventral flexure finishes, and the eyes and cerci are distinguished. (H) Egg at 56 dao. Katatrepsis occurs, and the secondary dorsal organ forms. (I) Egg at 63 dao. The embryo acquires its definitive form. Ab: abdomen, An: antenna, Ce: cercus, EA: embryonic area, EeA: extraembryonic area, E: eye, Em: embryo, H: head, InS: intercalary segment, Lr: labrum, L1: first thoracic leg, Pce: protocephalon, Pco: Protocorm, Sd: stomodaeum, SDO: secondary dorsal organ, Se: serosa, Th: thorax. Bars = 1 mm.





**Fig. 6.** Differentiation of the embryonic area (A) and germ disc or embryo (B) in *Metallyticus splendidus*, DAPI staining, fluorescence microscopy. A and B are the enlargements of Fig. 4A and B, respectively. See text. EA: embryonic area, EeA: extraembryonic area, Em: embryo, Se: serosa. Bars = 500  $\mu$ m.



**Fig. 7.** Diagrammatic representation showing the movement of the embryo in *Metallyticus splendidus*. The embryo undergoes blastokinesis of non-reversion type, involving the rotation around the egg's anteroposterior axis. See text.

### 3.2. Embryonic development

A blastoderm region with a high cellular density appears at the egg's ventral side, slightly biased toward the posterior (Figs. 4A, 5A, and 6A). This represents the newly differentiated embryonic area, where the blastoderm cells of the embryonic area simply concentrate further to form a circular, small germ disc or embryo approximately 400  $\mu$ m in diameter. The extraembryonic area is now called the “serosa” (Figs. 4B, 5B, 6B, and 7A). The embryo undergoes embryogenesis of a typical short germ band type (Figs. 4, 5, and 7). The formed embryo soon undergoes elongation, assuming a pear-shape with an anterior protocephalon and distinguished posterior protocorm (Figs. 4C and 7B). The embryo then extends to the ventral surface of the egg as the segmentation process begins (Figs. 4D and 5D). At the stage shown in Figs. 4E, 5E, and 7C, segmentation occurs in the cephalic and anterior thoracic

regions. During the stage shown in Figs. 4F, 5F, and 7D, segmentation proceeds to the third thoracic segment during which the appendages differentiate in the already differentiated segments and the stomodaeum appears. Figs. 4G and 5G show that the development has proceeded further, i.e., the segmentation is complete, the ventral flexure finishes, and the eyes and cerci are distinguished. In the middle stages of development, the most notable change is the rotation of the embryo (Figs. 5E–G and 7C–E); while maintaining its superficial position on the egg surface, the embryo shifts from the original ventral side of the egg (Figs. 5E and 7C) to the dorsal side (Figs. 5G and 7E) via the lateral side (Figs. 5F and 7D). The embryo further grows on the dorsal side of the egg and katatrepsis occurs, the secondary dorsal organ forms at the anterior part of the egg and definitive dorsal closure starts (Figs. 4H and 5H). The embryo then enters the final stage of embryogenesis, acquiring its definitive form (Figs. 4I, 5I, and 7F).

**Fig. 5.** Successive developmental stages of *Metallyticus splendidus*, DAPI staining, fluorescence microscopy, lateral views. The eggs in A–I are the same as those shown in Fig. 4A–I, respectively. See the text as well as the legend of Fig. 4. The embryo maintains its original orientation without reversing its anteroposterior direction and maintains its superficial position without being immersed in the yolk (A–I), but the embryo undergoes rotation around the egg's anteroposterior axis (E–G). Ab: abdomen, An: antenna, E: eye, EA: embryonic area, EeA: extraembryonic area, Em: embryo, H: head, L1: first thoracic leg, Sd: stomodaeum, SDO: secondary dorsal organ, Se: serosa, Th: thorax. Bars = 1 mm.

Throughout embryogenesis, the embryo maintains its original orientation without reversing its anteroposterior direction and maintains its superficial position without being immersed in the yolk (Figs. 4A–I, 5A–I, and 7A–F). The egg period of *M. splendidus* was 80–90 days at room temperature (18 °C–24 °C).

#### 4. Discussion

##### 4.1. Egg structure

The eggs of *M. splendidus* can be characterized by the following features: (1) spheroidal, with a pointed anterior pole, a convex ventral side, and a concave/straight dorsal side; (2) a chorion composed of exochorion and endochorion; (3) a hatching line along the coronal line at the anterior half of the egg; (4) funnel-shaped, grouped micropyles located in the central area on the ventral side of the egg; (5) numerous aeropyles along the hatching line and around the anterior pole of the egg. The listed features 1–3 are shared with the eggs of Mantidae, hitherto reported by Görg (1959) and Iwaikawa and Ogi (1982), and thus, may be regarded as the groundplan features of Mantodea.

Regarding feature 4, the present study of *M. splendidus* may be the first to describe mantodean micropyles, demonstrating the passage of the micropylar canal, which starts at the egg surface as a funnel-shaped micropyle, obliquely penetrates the exo- and endochorion and opens into the egg's inside. The present study reveals that many micropyles are grouped on the ventral side of the egg in *M. splendidus*. This distribution pattern of micropyles agrees with the earlier findings in Blattodea (Kadyi, 1879; Wheeler, 1889; Mukerji, 1970; review, Fujita and Machida, 2017). The grouped micropyles on the ventral side of the egg may be assumed to be a part of the groundplan of Dictyoptera, probably an apomorphic one, based on the fact that such a distribution has not been confirmed in other insects.

For *Tenoder* spp., Hagan (1917) described that a prominent button-shaped micropyle almost covers the anterior end of the egg, while Iwaikawa and Ogi (1982) described several micropyles arranged in a circle with one micropyle positioned at the center of them, at the anterior pole of the egg. However, no similar structure(s) identifiable as micropyle(s) were observed in the eggs of *M. splendidus* of Metallyticidae, a representative family of basal Mantodea, nor were they reported in the eggs of *Hierodula crassa* of Mantidae by Görg (1959). Therefore, the pore structures described in *Tenoder* were likely derived structures of this group.

Regarding feature 5, we observed numerous aeropyles along the hatching line and around the anterior pole of the egg in *M. splendidus*. Görg (1959) described numerous pores on the anterior pole of the egg in *Hierodula crassa* and identified them as “micropyles.” However, based on the resemblance in the distribution between “micropyles” described by Görg for *H. crassa* and the aeropyles we observed for *M. splendidus*; Görg's micropyles are probably the actual aeropyles.

##### 4.2. Embryonic development

We characterize the embryonic development of *M. splendidus* as follows: (1) the germ disc or embryo is formed by a simple concentration of blastoderm cells; (2) the embryo formed on the ventral side of the egg undergoes embryogenesis of the short germ band type; (3) the embryo retains its original, superficial position without being immersed in the yolk and maintains its original orientation without reversing its anteroposterior orientation during development; and (4) the embryo undergoes rotation around the anteroposterior axis of the egg in the middle stages of development.

Mashimo et al. (2014) reviewed the manner of embryo formation in non-holometabolous insects. They found that the embryo is formed by the fusion of paired blastoderm regions with high cellular density in Polyneoptera, i.e., in Phasmatodea (Bedford, 1970), Embioptera (Jintsu, 2010), Grylloblattodea (Uchifune and Machida, 2005), Zoraptera (Mashimo et al., 2014), Dermaptera (Shimizu, 2013), and Orthoptera (Miyawaki et al., 2004; Nakamura et al., 2010). Fujita and Machida (2017) also demonstrated this type of embryo formation for Blattodea. Based on the out-group comparison, Mashimo et al. (2014) suggested that embryo formation through the fusion of paired blastoderm regions with high cellular density is a potential autapomorphy of Polyneoptera. The manner of embryo formation in Mantodea is not well understood, and we have only a brief description without illustrating figures by Giardina (1897) on *M. religiosa*. He discussed that a U-shaped region with high cellular density first appears in the blastoderm and then a circular germ disc forms in *M. religiosa*. There is a possibility that the U-shaped region with high cellular density in *M. religiosa* is comparable with the paired blastoderm regions, which is regarded as polyneopteran potential autapomorphy. For better understanding of embryo formation in Mantodea, we closely examined embryo formation in the basal *M. splendidus*. However, the present study (feature 1 in the above list) revealed that the embryo is formed by a simple concentration of the blastoderm cells in *M. splendidus*, and neither the paired regions nor the U-shaped region with high cellular density was observed in the blastoderm. Critical and intensive examination is required to understand embryo formation in Mantodea, spanning its major lineages.

The features 2–4 listed above for *M. splendidus* are shared with the higher members of the mantodean family, Mantidae (Viallanes, 1891; Hagan, 1917; Görg, 1959); therefore, we may assume that they represent the groundplan of Mantodea. The feature 2, the embryogenesis of the short germ band type is predominantly found among non-holometabolous insects (Krause, 1939). The newly formed embryo in Mantodea, as shown in *M. splendidus*, is circular, without signs of an anterodorsal axis or predetermined segmentation, and the short germ band type in Mantodea represents the most typical one.

As suggested by Fujita and Machida (2017), embryos of non-holometabolous insects almost exclusively undergo blastokinesis, which involves reversion of the embryo's anteroposterior axis (i.e., the “reversion type”). In contrast, blastokinesis without involving the reversion of the embryo's anteroposterior ends (“non-reversion type”) has not been reported in polyneopterans and other non-holometabolous insects other than the blaberoidean Blattodea and the Mantodea, as confirmed for *M. splendidus* in the present study (the feature 3 in the above list). Thus, the “non-reversion type” of blastokinesis may be regarded as derived type. However, we have no information that allows us to determine whether the sharing of “non-reversion type” by Mantodea and blaberoidean Blattodea is because of homology or homoplasy (cf. Fujita and Machida, 2017).

Embryo rotation is known to occur in another member of Dictyoptera, the Isoptera (Klower, 1900; Striebel, 1960). However, embryo rotation in Isoptera differs from that in Mantodea, in that it occurs in the final stage of development. Furthermore, the rotation sporadically occurs in various other insect groups other than Mantodea and Isoptera, e.g., Odonata (Miyakawa, 1987), Orthoptera (Pétavy, 1985), Phasmatodea (Fournier, 1967; Bedford, 1970) [strictly in Euphasmatodea because Timematodea embryos do not undergo rotation (Jintsu et al., 2010)], Hemiptera (Cobben, 1968), and Lepidoptera (Presser and Rutschky, 1957). Embryo rotation is likely to have evolved independently in several lineages, including Mantodea.

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