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Eggs and embryonic development of the snakefly *Mongoloraphidia harmandi* (Insecta: Neuropterida, Raphidioptera, Raphidiidae)

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ABSTRACT

We examined and described the eggs and several developmental stages of the preblastokinesis period of *Mongoloraphidia harmandi* (Raphidiidae) and compared the information obtained with that of other Neuropterida. Similar to other Raphidioptera, the eggs of *M. harmandi* are cylindrical, with a well-developed micropylar projection at the anterior pole of the egg. The micropylar projection at the anterior pole of the egg is regarded as the groundplan of Neuropterida. Several developmental stages of the preblastokinesis period comparable to those in *Inocellia japonica* (Inocelliidae) could be identified in *M. harmandi*. As in *I. japonica*, the blastoderm concentrates midway through the egg, forming a wide belt with a higher cellular density, followed by the formation of an elongated embryo. The embryonic development observed in Raphidioptera, involving the formation of a belt-like, condensed blastoderm, has not been observed in any other insects, and may thus be considered an autapomorphy of Raphidioptera. The resulting long embryo undergoes segmentation almost synchronously. Thus, the embryogenesis of *M. harmandi* is categorized as the long-germ type, as in other Neuropterida, including *I. japonica*. In members of the Holometabola and Neuropterida that undergo long-germ embryogenesis, including *I. japonica*, embryonic elongation during the preblatokinesis period occurs on the egg surface. In contrast, the *M. harmandi* embryo immerses into the egg and localizes itself along the anteroposterior axis of the egg, exhibiting the embryogenesis of the immersion type. This is unusual among insects that undergo long-germ type embryogenesis and can thus be regarded as an autapomorphy of *M. harmandi*.

KEYWORDS comparative embryology, blastokinesis, micropylar projection, Neuropterida, germ type, Megaloptera, Neuroptera

1. INTRODUCTION

Neuropterida consists of the orders Neuroptera (Planipennia), Megaloptera, and Raphidioptera, each of which is highly specialized, and is a very interesting group in insect phylogeny. While the relationships among these three orders have been much investigated, the current prevailing view may be "Raphidioptera + (Megaloptera + Neuroptera)" (e.g., Aspöck et al. 2012; Wang et al. 2017; Engel et al. 2018). Characterizing the Neuropterida ideally requires an examination from multiple perspectives. Raphidioptera (snakeflies), which represents the sister group of Megaloptera + Neuroptera, is a small group (about 240 extant species, Haring et al. 2011) but crucial in reconstructing the evolution and groundplan of Neuropterida. Thus, we conducted embryological studies of Raphidioptera, which contains two families, Inocelliidae and Raphidiidae, using *Inocellia japonica* Okamoto, 1917 belonging to Inocelliidae (Tsutsumi and Machida 2004, 2006).

In that study, we obtained a female of *Mongoloraphidia* (*Japanoraphidia*) *harmandi* (Navás, 1909) of Raphidiidae, from which we collected about 50 eggs. This allowed us to gain a deeper understanding of the egg and embryology of Raphidioptera by uncovering that the blastokineses of *I. japonica* and

M. harmandi differ significantly in the localization of embryos during the preblastokinesis period. However, Tsutsumi (2006) and Tsutsumi et al. (2019) only provided brief notes on this. In this paper, we report in detail our findings on the egg and embryology of *M. harmandi* from our previous studies (Tsutsumi 2006; Tsutsumi et al. 2019), compare them with those of other Raphidioptera and Neuropteroidea, and discuss them in a phylogenetic context.

2. MATERIALS AND METHODS

One female of *Mongoloraphidia harmandi* collected at Yabuhara, Kiso, Nagano Prefecture, Japan (ca. 1,000 m alt.) in June 22, 2005 (for details, Tsutsumi 2006) was reared and fed on aphids. The deposited eggs were fixed in Karnovsky's fixative (2% paraformaldehyde + 2.5% glutaraldehyde 0.1M HCl–sodium cacodylate buffer solution, pH 7.2). A portion of eggs, of which chorion was punctured with a fine needle, were stained for 1 week with DAPI solution (4',6-diamidino-2-phenylindole dihydrochloride, diluted to approximately 5 µg/ml with distilled water) and observed under a fluorescence stereomicroscope (MZ FL III + FLUO COMBI, Leica, Heerbrugg, Switzerland) with UV excitation (360 nm). Other eggs were postfixed with 1% OsO_4 for 30 min, dehydrated through a graded ethyl alcohol series, dried in a critical point dryer, coated with gold, and observed under a scanning electron microscope (SM-300, TOPCON, Tokyo, Japan).

3. RESULTS

3.1. Eggs

Mongoloraphidia harmandi eggs are light-colored, cylindrical, slightly tapered posteriorward, and approximately 1.1 mm long and 200 µm thick (Fig. 1A). At the anterior pole of the egg is a short cylindrical knob approximately 60 µm in height and 50 µm in diameter. The surface of the micropylar projection consists of irregularly shaped facets (Fig. 1B). Approximately 15 micropyles were found around the base of the micropylar projection.

3.2. Embryonic development

Tsutsumi and Machida (2006) described the embryonic development of *Inocellia japonica*, dividing it into nine stages: six preblastokinesis (Stages 1–6) and three postblastokinesis stages (Stages 7–9). The *M. harmandi* eggs examined in the present study are shown in Fig. 2A–F, all of which represent the preblastokinesis stages. In the present study, we describe the embryological features of *M. harmandi*, comparing them with those of *I. japonica*. The developmental stages of *M. harmandi* eggs shown in Fig. 2A–F correspond to those defined for *I. japonica* by Tsutsumi and Machida (2006) as the early Stage 2, early

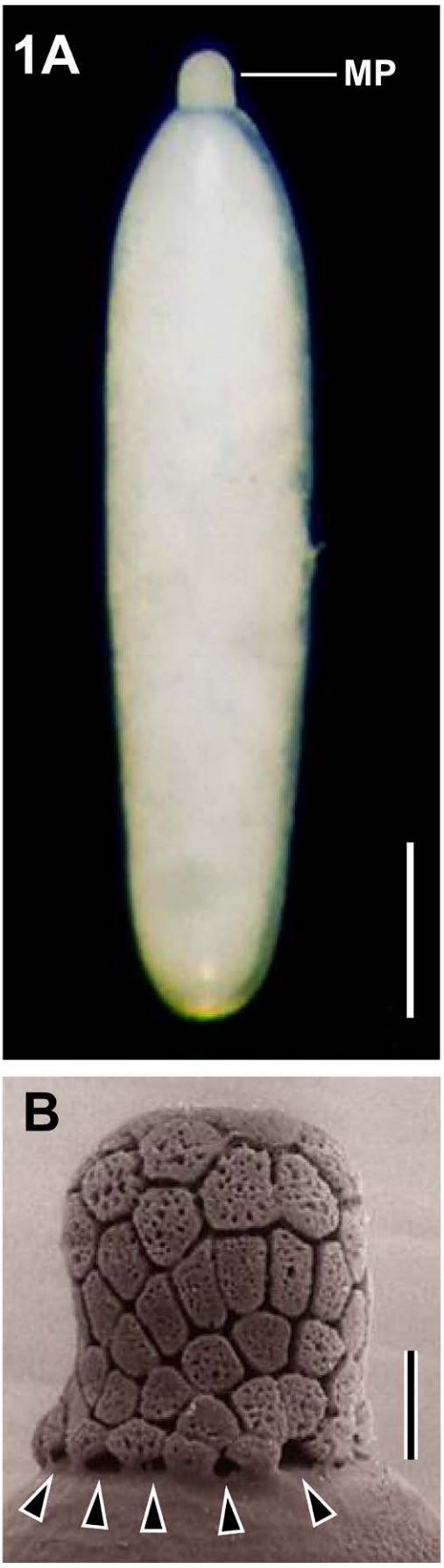

Fig. 1 Eggs of *Mongoloraphidia harmandi*. A. Egg. B. A scanning electron micrograph of the micropylar projection. MP: micropylar projection. Arrowheads indicate micropyles. Scales = A: 200 µm; B: 20 µm.

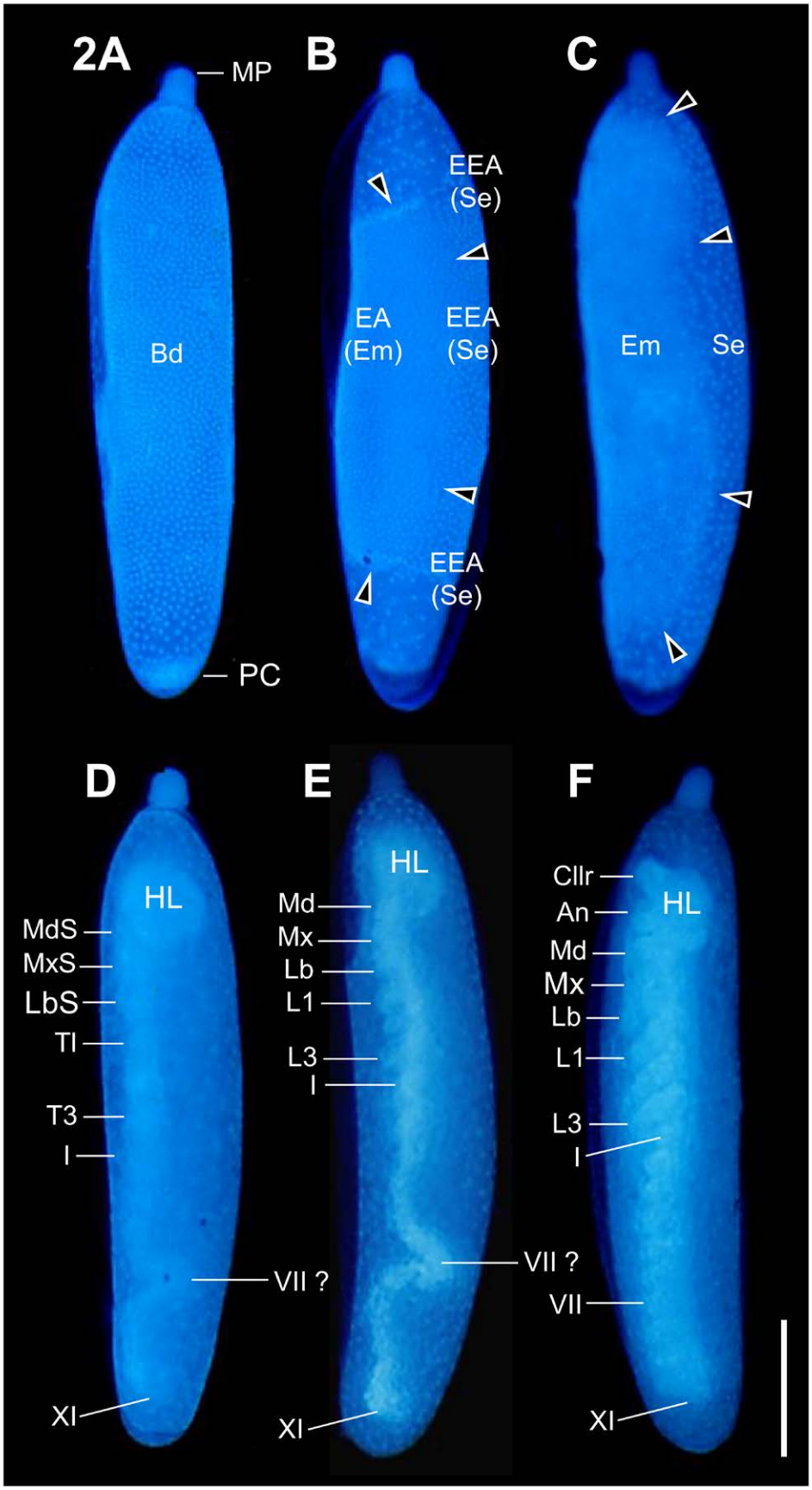

Fig. 2 *Mongoloraphidia harmandi* eggs at different developmental stages, fluorescence microscopy. Lateral views, ventral to the left. A. Early Stage 2; B. early Stage 3; C. Middle to late Stage 3; D. Stage 4; E. Stage 5; F. Stage 6. An: antenna, Bd: blastoderm, Cllr: clypeolabrum, EA: embryonic area, EEA: extraembryonic area, Em: embryo, HL: head lobe, L1, 3: first and third thoracic legs, Lb: labium, LbS: labial segment, Md: mandible, MdS: mandibular segment, MP: micropylar projection, Mx: maxilla, MxS: maxillary segment, PC: pole cell, Se: serosa, T1, 3: first and third thoracic segments, I–XI: first to 11th abdominal segments. Arrowheads indicate the expansion of the embryonic area or embryo. Scale = 200 μm.

Stage 3, middle to late Stage 3, Stage 4, Stage 5, and Stage 6, respectively.

The egg shown in Fig. 2A

In *I. japonica*, a uniform blastoderm is formed early in Stage 2 (Tsutsumi and Machida 2006, figs 1A, 3). The blastoderm cells gradually concentrate midway through the egg to form a wide belt with a higher cellular density encircling the middle of the egg, which is called the "condensed blastoderm" in late Stage 2 (Tsutsumi and Machida 2006, figs 1B, 4). In the *M. harmandi* egg shown in Fig. 2A, a newly formed uniform blastoderm is observed, corresponding exactly to early Stage 2 in *I. japonica*. As in the *I. japonica* egg at early Stage 2 (Tsutsumi and Machida 2006, figs 1A, 3), pole cells are seen at the posterior pole of the egg (Fig. 2A). The condensed blastoderm as described in late Stage 2 of *I. japonica* was not found in *M. harmandi*. Notably, however, in the egg shown in Fig. 2B, a wide belt-like area with a higher cellular density, which resembles the condensed blastoderm in *I. japonica*, could be clearly identified. Therefore, the concentration of blastoderm midway through the egg found in *I. japonica* can be safely assumed to also occur in *M. harmandi*.

The eggs shown in Fig. 2B and 2C

In *I. japonica*, the embryo is formed in Stage 3. First, regional differentiation occurs in the condensed blastoderm. Namely, its ventral half becomes more densely cellulated, differentiating into the embryonic area, and the remaining region is the extraembryonic area (Tsutsumi and Machida 2006, figs 1C, 5). Subsequently, the embryonic area expands anteriorly and posteriorly to eventually form the elongated embryo that reaches the anterior and posterior poles of the egg, with the extraembryonic area becoming the serosa (Tsutsumi and Machida 2006, fig. 1D).

In the *M. harmandi* egg shown in Fig. 2B, the ventral half of the belt-like area is densely cellulated, indicating that the egg is at the stage of the differentiation of embryonic and extraembryonic areas. In the egg in Fig. 2C, the differentiated embryo elongates, with its anterior and posterior ends nearly reaching both poles of the egg. Therefore, the egg shown in Fig. 2B corresponds to early Stage 3 in *I. japonica*, while the egg in Fig. 2C likely represents middle to late Stage 3 in *I. japonica*, during which the embryonic region elongates.

The egg shown in Fig. 2D

In *I. japonic*a, embryonic segmentation occurs in Stage 4. The newly formed embryo is segmented almost synchronously throughout its whole length, and the several cephalic, three thoracic, and 11 abdominal segments are complete (Tsutsumi and Machida 2006, figs 1E, 7A, B). In the *M. harmandi* egg shown in Fig. 2D, segmentation rapidly occurs, forming the segments up to the 11th abdominal one, which corresponds to Stage 4 in *I. japonica*.

However, significant differences emerge between the embryogeneses of *I. japonica* and *M. harmandi* at Stage 4 regarding the embryo posture and localization in the egg. In *I. japonica*, the embryo lies along the egg surface as it elongates, with the posterior end of the embryo reaching the dorsal surface of the egg beyond the posterior pole of the egg (Tsutsumi and Machida 2006, figs 1E, 7A, B). In *M. harmandi*, however, the embryo gradually immerses into the egg inside as it develops. Its posterior end does not progress beyond the posterior pole toward the dorsal side of the egg; instead, the embryo itself curves dorsad at approximately the seventh abdominal segment (Fig. 2D).

The egg shown in Fig. 2E

In Stage 5, the *I. japonica* embryo continues to elongate along the egg surface, with its caudal end further migrating toward the anterior pole of the egg, attaining its maximum length during the preblastokinesis period, and the appendages develop (Tsutsumi and Machida 2006, figs 1F, 8A, B). In the *M. harmandi* egg shown in Fig. 2E, the embryo becomes more deeply immersed within the egg, aligning itself along the anteroposterior axis of the egg. As it elongates, its abdominal curvature deepens, and the embryo reaches its maximum length during the preblastokinesis period. The appendages develop. Thus, the developmental stage of the *M. harmandi* egg in Fig. 2E corresponds to Stage 5 in *I. japonica*.

The egg shown in Fig. 2F

In *I. japonica*, the embryo contracts in Stage 6. The rear end of the abdomen, which had occupied the dorsal side of the egg in earlier stages, migrates to the ventral side, causing the embryo to straighten (Tsutsumi and Machida 2006, figs 1G, 9). This is the final stage in the preblastokinesis period, as the blastokinesis (strictly, katatrepsis) occurs subsequently in Stage 7 (Tsutsumi and Machida 2006, fig. 1H). Throughout the preblastokinesis period, the *I. japonica* embryo develops on the egg surface, categorizing its embryogenesis as the "superficial type".

In the *M. harmandi* egg shown in Fig. 2F, the embryo has also contracted, and its abdominal curvature has resolved, resulting in a straight embryo localized along the anteroposterior axis of the egg. Because the *M. harmandi* embryo develops inside the egg during the preblastokinesis period, its embryogenesis is categorized as the "immersion type". Although such eggs have not been obtained, the embryo would eventually emerge onto the egg surface through blastokinesis (katatrepsis).

The developmental stage of the *M. harmandi* egg shown in Fig. 2F is thus comparable to Stage 6 in *I. japonica*.

DISCUSSION

4.1. Egg

Mongoloraphidia harmandi (Raphidiidae) eggs exhibit a cylindrical oval shape and a well-developed micropylar projection at the anterior pole. These features are shared with eggs of *Inocellia japonica* (Inocelliidae) (Tsutsumi and Machida 2004, figs 1, 2, 2006, fig. 2A, B) and other Raphidioptera (Woglum and McGregor 1959; Aspöck and Aspöck 1991; Kubrakiewicz et al. 2005) and are regarded as the groundplan of Raphidioptera.

The eggs of the other two orders in Neuropterida, i.e., Megaloptera and Neuroptera, also feature a micropylar projection at the anterior pole (Hinton 1981; Kubrakiewicz et al. 2005), and this is regarded as the groundplan of Neuropterida. Megaloptera consists of two families, Corydalidae and Sialidae, both of which have a well-developed micropylar projection (Corydalidae: Barker and Neunzig 1968; Miyakawa 1979; Kubrakiewicz et al. 2005; Sialidae: Matsuzaki and Ando 1977; Suzuki et al. 1981). Micropylar projections at the anterior pole of the egg are basically present in Neuroptera (see Hinton 1981; Kubrakiewicz et al. 2005), although sometimes less developed and appearing low or plate-like (Hinton 1981). Furthermore, in Myrmeleonidae (Withycombe 1925; Kubrakiewicz et al. 2005) and Ascalaphidae (Withycombe 1925; Kamiya and Ando 1985), the micropyles are merely arranged in a ring without forming a distinct projection, and an additional micropylar region is present at the posterior pole. These exceptional micropylar characteristics in Neuroptera can be considered as derived, in line with the current phylogenetic consensus that the Neuroptera is a more derived Neuropterida and the Myrmeleonidae and Ascalaphidae are more derived lineages in Neuroptera (e.g., Aspöck et al. 2012; Wang et al. 2017; Engel et al. 2018). In contrast, the well-developed micropylar projection at the anterior pole of the egg, such as in Raphidioptera and Megaloptera can be understood to be ancestral in Neuropterida.

4.2. Embryonic development

In *I. japonica* (Inocelliidae), the blastoderm concentrates midway through the egg, forming a wide belt with a higher cellular density or the condensed blastoderm, from which an elongated embryo develops (Tsutsumi and Machida 2006). In the present study, a similar mode of embryogenesis was observed in *M. harmandi* (Raphidiidae). The formation of embryo accompanied by the formation of a belt-like condensed blastoderm, has not been observed in any other insect orders and may therefore represent an autapomorphic trait of Raphidioptera.

The elongated embryos of Raphidioptera undergoing this mode of development soon undergo segmentation, with all segments differentiating almost simultaneously (Tsutsumi and Machida 2006; herein). Accordingly, raphidiopteran embryogenesis can be categorized as the long-germ type (cf. Krause 1939; Sander 1976). Embryogenesis in other Neuropterida is also invariably of long-germ type (e.g., Megaloptera: Miyakawa 1979; Ando et al. 1985; Neuroptera: Bock 1939; Kamiya and Ando 1985), which is predominant in Holometabola (Anderson 1972a, b). During the preblastokinesis period, the embryos of Neuropterida, including *I. japonica*, like the other embryos showing the embryogenesis of long-germ type (Anderson 1972a, b; coleopteran Archostemata is exceptional, as touched on below), elongate on the egg surface (superficial type), with their caudal end often extending beyond the posterior pole and reaching the dorsal surface of the egg (inocelliid Raphidioptera: Tsutsumi and Machida 2006; Megaloptera: Miyakawa 1979; Ando et al. 1985; Neuroptera: Bock 1939). Within Neuropterida, *M. harmandi* represents a notable exception, as demonstrated in the present study. Its embryo immerses into the egg during the preblatokinesis period and aligns itself along the anteroposterior axis, thus exhibiting immersion-type embryogenesis. Given that the embryogenesis in other Neuropterida is of the superficial type, that Inocelliidae including *I. japonica* is regarded as the sister group to all remaining, diverged Raphidioptera including *M. harmandi* (Haring et al. 2011), and that *I. japonica* exhibits the superficial type whereas *M. harmandi* the immersion one, the immersion type in *M. harmandi* can be interpreted as an autapomorphy of this species.

We (Kojima et al. in submission) explored a parallel case in the embryonic development of *Tenomerga mucida* (Chevrolat, 1829) of Archostemata (Coleoptera). We found that *T. mucida* exhibits immersion-type embryogenesis. Thus, like *M. harmandi*, also this mode of development is considered an autapomorphic trait of the group.

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