

Embryonic Development of a Snakefly, *Inocellia japonica* Okamoto: An Outline (Insecta: Neuroptera, Raphidioidea)

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Abstract

An outline of the embryonic development of a snakefly, *Inocellia japonica* is described. Typical superficial cleavage gives rise to a cellular layer, the blastoderm, the posterior cells of which differentiate into pole cells. Cells of the newly formed blastoderm around the anterior and posterior parts of the egg concentrate midway through the egg, to form a wide belt with a high cellular density encircling the middle of the egg, which is named the “condensed blastoderm.” Blastoderm cells around the anterior and posterior parts of the egg which did not participate in the formation of the condensed blastoderm sink into the yolk to become secondary yolk cells. The cells of the lateral to ventral sides of the condensed blastoderm become more densely packed and differentiate into an embryonic area, and accompanied by anterior- and posteriorward extensions of the area, the germ band forms. The amnioserosal folds form soon after the formation of the germ band, and anatrepsis completes prior to the commencement of segmentation. The germ band or the embryo undergoes embryogenesis of the long germ band type. The embryo retains its original position on the ventral side of the egg throughout embryogenesis, although its rear part extends to the dorsal side of the egg in the longest-embryo stage, and the embryogenesis is of the typical superficial type. Eleven segments differentiate in the abdomen, although the tenth and eleventh ones fuse into a single segment in the later stage. The segmental appendages develop in the first to ninth and eleventh abdominal segments: the appendages in the first abdominal segment are the pleuropodia. The clypeolabrum differentiates as paired structures, which are observed to initially associate with the intercalary segment.

The formation of the germ band accompanied by the condensation of the blastoderm and early completion of anatrepsis are notable embryological characteristics of Raphidioidea among Neuroptera.

Introduction

Neuroptera, which represent one of the most basal clades in Holometabola, are one of the key groups for reconstructing the groundplan and elucidating the origin and evolution of Holometabola. Although the intersubordinal relationships comprising Neuroptera, *i.e.*, Raphidioidea, Planipennia and Megaloptera, have been highly controversial, recent phylogenetical studies on Neuroptera (*e. g.*, Aspöck *et al.*, 2001; Aspöck, 2002; Haring and Aspöck, 2004) often bestow the most basal position within Neuroptera to Raphidioidea, so that a comprehensive understanding of Raphidioidea has become more desirable than ever. A comparative embryological approach is one of the most useful methods for phylogenetic discussions, but we have no embryological information on Raphidioidea, although there are several works with respect to other neuropteran suborders, Planipennia (Bock, 1939, 1941; Kamiya and Ando, 1985) and Megaloptera (Strindberg, 1915; Du Bois, 1936, 1938; Miyakawa, 1979, 1980; Suzuki *et al.*, 1981; Ando *et al.*, 1985).

For this reason, we have started an embryological study of Raphidioidea using a snakefly, *Inocellia japonica*, as materials, aiming at a full illustration of raphidiodean embryogenesis and a novel reconstruction of the groundplan of Neuroptera and Holometabola from an embryological basis. As a first step, in this paper, we outline the embryogenesis

of *Inocellia japonica*.

Materials and Methods

Females of *Inocellia japonica* Okamoto were collected at Tsukuba, Ibaraki prefecture, and Ueda, Nagano prefecture, in May to July of 2002 to 2005. Females were reared separately, fed with honey, at room temperature (about 18°C) in a plastic case (10 cm×10 cm×3 cm), in which some pieces of bark of a Japanese red pine, *Pinus densiflora*, were put as oviposition beds.

Laid eggs were incubated under room temperature (about 18°C), fixed in Bouin's (12 h) or Karnovsky's (24 h) fixative, in which eggs were punctured with a fine needle, and respectively stored in 70% ethyl alcohol or 0.1 M sodium cacodylate buffer. Stored eggs were hydrated through a graded ethyl alcohol series, stained a week with DAPI solution [*N*-(4',6-diamidino-2-phenylindole) dihydrochloride, diluted about 10 µg/l with phosphate-buffered saline (PBS; 18.6 mM NaH₂PO₄·H₂O, 84.1 mM NaH₂PO₄·2H₂O, 1.75 M NaCl; pH 7.4)], and observed under a Leica MZ FL III fluorescence stereomicroscope with UV excitation (360 nm). For the observation of the external egg structure, the fixed eggs were postfixed with 1% OsO₄ for 5 min, dehydrated through a graded ethyl alcohol series, and then transferred to acetone. They were dried in a critical point dryer (TOPCON CP-5A), coated with gold, and observed under an SEM (TOPCON SM-300).

Results

Egg (Figs. 2A, B)

The eggs of *Inocellia japonica* were deposited under bark of the tree as a batch of about 50 eggs. The eggs are cylindrical with 1.1 mm long and 0.2 mm short diameters (Fig. 2A), and yellowish in color, the inside yolk being visible through a transparent chorion. At the anterior pole of the egg is a knob-like projection, the "micropylar projection," the surface of which is porous and assumes a complex pattern (Fig. 2B). Around the base of the micropylar projection about 20 micropyles open (Fig. 2B) (see Tsutsumi and Machida, 2004).

Embryonic development

The egg period of *Inocellia japonica* ranges from 7 to 9 days at room temperature (about 18°C). Herein, we describe the embryonic development, dividing it into nine stages.

Stage 1

Fertilization occurs at the level of a third of the egg long diameter from the anterior egg pole. Egg cleavage is of a typical superficial type.

Stage 2 (Figs. 1A, B, 3, 4)

At about 14 h after oviposition, cleavage nuclei reach the egg surface and repeatedly divide there, and a unicellular layer covering the entire surface of the egg or the blastoderm forms. The blastoderm cells occupying the posterior egg pole are larger and rounded, to differentiate into pole cells (Figs. 1A, 3).

Cells of the newly formed blastoderm around the anterior and posterior parts of the egg soon start to concentrate midway through the egg, to form a wide belt with high cellular density encircling the middle of the egg, which is named the "condensed blastoderm" (Figs. 1B, 4).

Stage 3 (Figs. 1C, D, 5, 6)

The regional differentiation occurs in the condensed blastoderm (Figs. 1C, 5). The cells of lateral to ventral sides of the condensed blastoderm become more densely packed and differentiate into an embryonic area or the germ rudiment. The cells occupying a narrow dorsal area of the condensed blastoderm differentiate into an extraembryonic area or the serosa. Simultaneously, the cells of the germ rudiment, condensing ventralwards, start to migrate and extend towards the anterior and posterior. The blastoderm cells around the anterior and posterior parts of the egg which had not participated in the formation of the condensed blastoderm sink into the yolk, forming some clusters of cells, to become the secondary yolk cells (Figs. 1C, 5).

The germ rudiment's extension anterior and posterior proceeds until finally the anterior and posterior poles of the

egg are reached. Simultaneously, the primitive groove appears along the midline of the germ rudiment (Fig. 6), leading to mesodermal segregation (Tsumumi and Machida, in preparation), and the germ band or the embryo is completed, comprising a broad anterior protocephalon and a narrower, elongated protocorm (Fig. 1D).

Parallel with the germ band's formation, the amnioserosal folds form. Due to the fusion of the amnioserosal folds, the embryo is ventrally covered by the amnion and serosa (Fig. 1D).

Stage 4 (Figs. 1E, 7A, B)

Soon after the completion of the germ band, the segmentation commences. At first, the mesothoracic segment differentiates, and the segmentation sequentially but rapidly proceeds towards the anterior and posterior. In the gnathal region, the mandibular, maxillary, and labial segments form, just anterior to the mandibular segment, the intercalary segment develops, in the protocephalic region, the antennal segment forms, in the thoracic region, three thoracic segments arise, and in the abdominal region, 11 segments form (Fig. 1E). Due to the posteriorward elongation of the embryo, the ninth abdominal segment is located at the posterior pole of the egg, and the tenth and eleventh abdominal segments take their positions on the dorsal side of the egg: the embryo assumes a J-shape (Figs. 1E, 7B).

The segmentation proceeds rapidly and almost simultaneously throughout the length of the embryo in *Inocellia japonica*, and the embryo of this insect can be categorized as being of the long germ band type. The embryo takes a superficial position on the egg's surface, even in the diapause stage when it is concealed by the amnion and serosa, and the localization of the *Inocellia japonica* embryo is of a superficial type. In the segments that differentiated earlier; *i. e.*, the antennal, gnathal, and thoracic segments, a pair of appendages forms (Figs. 1E, 7A). No appendages develop in the intercalary segment.

Stage 5 (Figs. 1F, 8A, B)

The embryo attains its maximum length at this stage (Fig. 1F). The appendages that have already differentiated elongate, and a pair of appendages or the pleuropodia form in the first abdominal segment (Figs. 1F, 8A). The stomodaeum appears anterior to the intercalary segment as a shallow pit (Fig. 8A). A pair of clypeolabral anlagen arise in front of the stomodaeum: they have an association with the intercalary territory, and their posterior elongations appear to make contact with each other behind the stomodaeum (Fig. 8A). The proctodaeum appears in the eleventh abdominal segment (Fig. 8B).

Stage 6 (Figs. 1G, 9)

The embryo contracts, and the rear of the abdomen, which has occupied the dorsal side of the egg, migrates to the ventral side of the egg, so that the embryo becomes straight (Figs. 1G, 9).

Cephalic appendages further elongate, and the palp differentiates in the maxilla (Figs. 1G, 9). Thoracic appendages further elongate, in which articulation commences (Fig. 1G). A pair of appendicular swellings form on the lateroventral side of each of the second to ninth abdominal segments (Fig. 1G). In the tenth abdominal segment, no signs of appendages are found, and in the eleventh abdominal segment, a pair of tiny appendicular elevations is temporarily observed. A columnar protrusion appears and elongates at the center of the pleuropodium. The stomodaeum is no longer observed, being covered by the growing cephalic appendages (Fig. 9). Paired spiracles appear at the anterolateral sides of the mesothoracic, metathoracic, and first eight abdominal segments.

Stage 7 (Figs. 1H, 10)

Katatrepsis occurs. The amnioserosal folds are withdrawn, the amnion appears on the egg's surface, and the serosa starts to move and to concentrate towards the dorsal side of the egg. The area from which the serosa has regressed is now occupied by the amnion, which functions as a provisional dorsal closure (Fig. 1H).

Paired clypeolabral anlagen fuse together into a single structure (Fig. 10). Cephalic and thoracic appendages further develop (Fig. 1H). The central protrusion of the pleuropodium invaginates. The tenth and eleventh abdominal segments fuse, and the abdominal segments appear to number 10 (Figs. 1H, 10).

Stage 8 (Figs. 1I, 11)

The body walls grow upwards and the definitive dorsal closure proceeds from anterior and posterior. Serosal cells

are condensed at the back of the first and second abdominal segments into a discoidal structure, the secondary dorsal organ (Fig. 1I). Cephalization proceeds extensively (Fig. 1I). Thoracic appendages acquire their definitive structures. The abdominal appendicular swellings other than the first abdominal ones or pleuropodia degenerate and disappear. The terminal abdominal segment or fused tenth and eleventh abdominal segments begin to bend ventrally (Fig. 1I).

Stage 9 (Figs. 1J, 12)

Replacing the provisional dorsal closure or the amnion, the definitive dorsal closure is completed (Fig. 1J), and the secondary dorsal organ sinks beneath the definitive dorsal closure, to degenerate inside the embryo's body. The

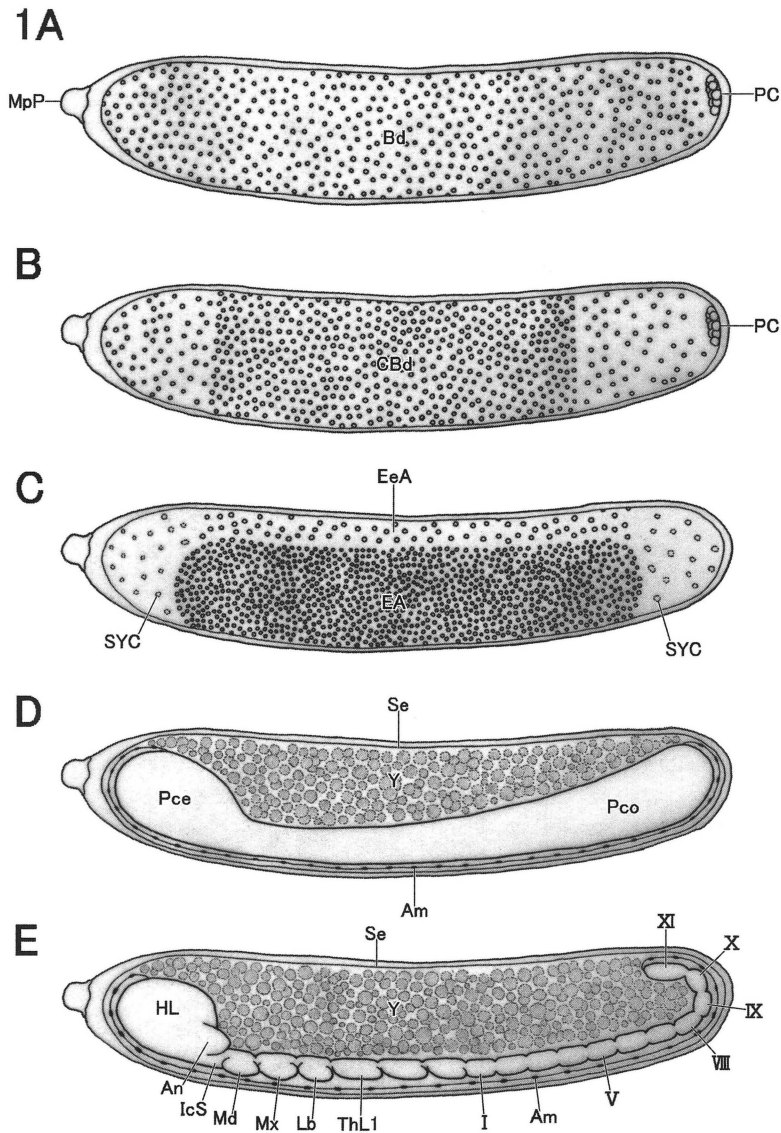


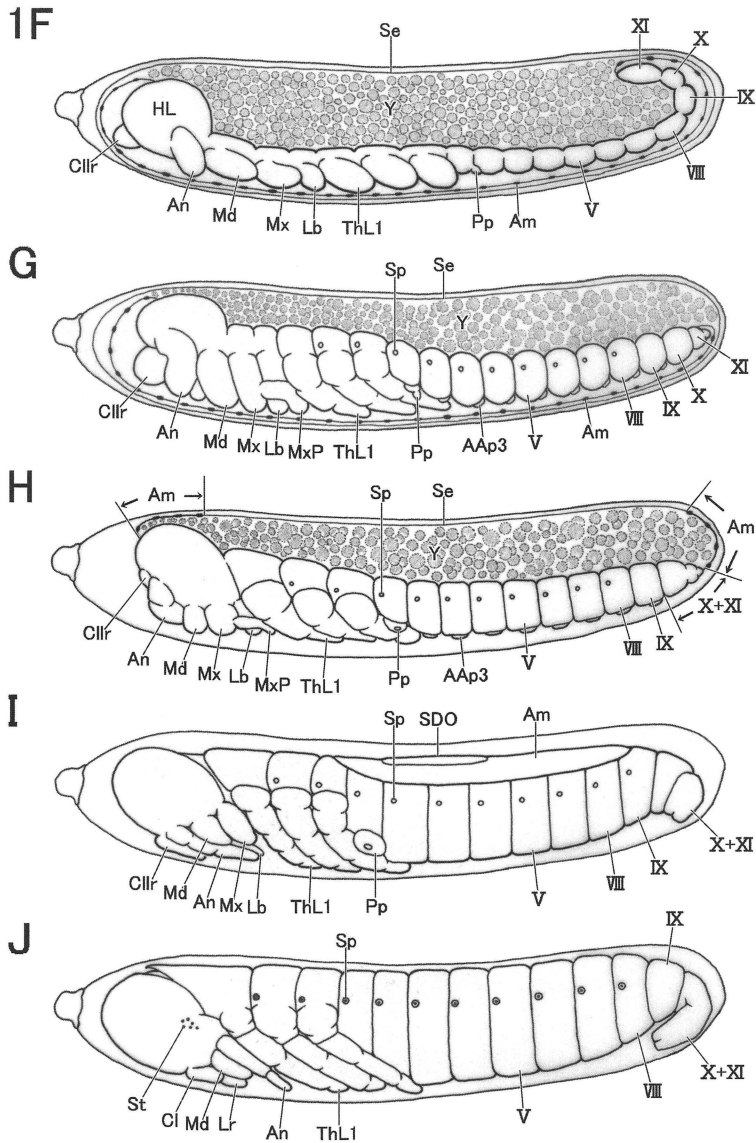
Fig. 1 Embryonic development of *Inocellia japonica*. Lateral views. A, B. Early (A) and late (B) Stage 2. C, D. Early (C) and late (D) Stage 3. E. Stage 4. F. Stage 5. G. Stage 6. H. Stage 7. I. Stage 8. J. Stage 9. AAP3: third abdominal appendage, Am: amnion, An: antenna, Bd: blastoderm, CBd: condensed blastoderm, Cl: clypeus, Clr: clypeolabrum, EA: embryonic area, EeA: extra-embryonic area, HL: head lobe, IcS: intercalary segment, Lb: /

embryo acquires the configuration of the first instar larva (Figs. 1J, 12). Stemmata appear as red spots near the base of the antenna: they are represented only by pigmentation, without the differentiation of lenses.

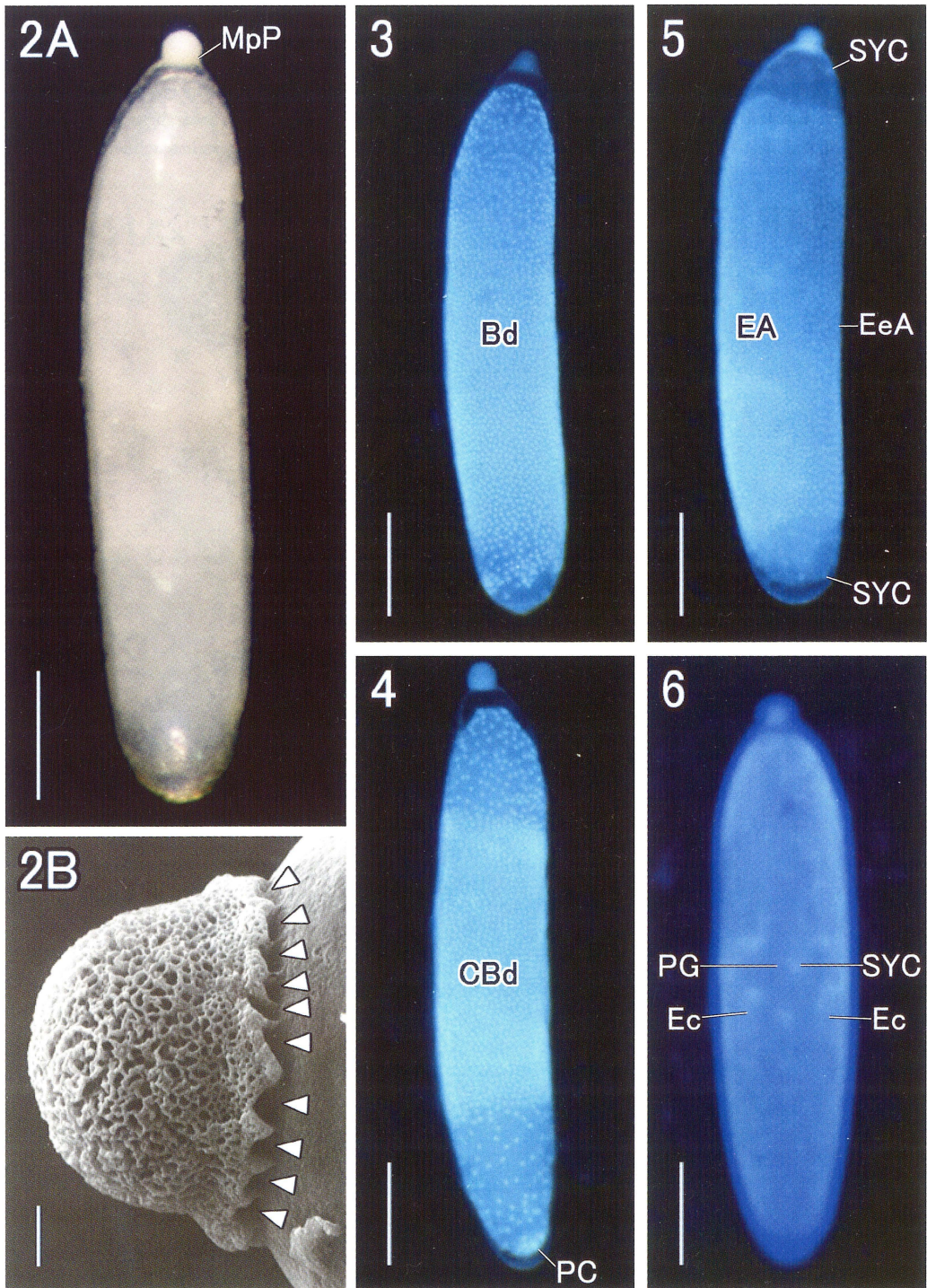
Discussion

Formation of the germ band

The embryo of *Inocellia japonica* is of the long germ band type. Generally, in insects with an embryo of this type, the germ band is directly derived from a presumptive embryonic area of the blastoderm roughly as extensive as itself (see Johannsen and Butt, 1941; Anderson, 1972b). In *Inocellia japonica*, however, a peculiar process of substantial condensation of the blastoderm is involved in the formation of the germ band. This process has neither been reported



∟ labium, Lr: labrum, Md: mandible, MpP: micropylar projection, Mx: maxilla, MxP: maxillary palp, PC: pole cell, Pce: protocephalon, Pco: protocorm, Pp: pleuropodium, SDO: secondary dorsal organ, Se: serosa, Sp: spiracle, St: stemma, SYC: secondary yolk cell, ThL1: prothoracic leg, Y: yolk, I–XI: first to eleventh abdominal segments.



Figures 2-6

in other neuropterans (Strindberg, 1915; Du Bois, 1936, 1938; Bock, 1939, 1941; Miyakawa, 1979, 1980; Suzuki *et al.*, 1981; Ando *et al.*, 1985; Kamiya and Ando, 1985), nor in other insects with embryogenesis of the long germ band type (see Johannsen and Butt, 1941; Anderson, 1972b).

The evolutionary change from the "hemimetabolous" short germ band type to the long germ band type, which is thought to be the groundplan of Holometabola, is one of the most attractive issues in insect comparative embryology. However, it is only speculation that the embryo of the long germ band type evolved from that of the short germ band type *via* a semi-long germ band type (cf. Krause, 1939; Sander, 1984). In this respect, the present finding on the germ band of *Inocellia japonica* may be of interest. The development of the germ band accompanied by the condensation of the blastoderm observed in this insect reminds us of the formation of embryos of the short germ band type predominant to hemimetabolous insects, which involves an extensive concentration of blastoderm cells (see Johannsen and Butt, 1941; Anderson, 1972a). Taking into account that Raphidioidea represent one of the basal clades of Holometabola, it is probable that the condensation of the blastoderm in *Inocellia japonica* is a remnant of the concentration of blastoderm cells in the formation of embryos of the short germ band type.

Amnioserosal fold

In *Inocellia japonica*, the amnioserosal folds close and anatrepsis is completed prior to the commencement of segmentation. Anatrepsis finishes much earlier in Raphidioidea than other neuropterans. In Megaloptera, the segmentation and anatrepsis proceed simultaneously, and the anatrepsis is completed when the segmentation has occurred up to the fifth and sixth abdominal segments in the dobsonfly *Protohermes grandis* (Miyakawa, 1979). In Planipennia, the amnioserosal folds are not developed until the abdominal segments form in the owlfly *Ascalaphus ramburi* (Kamiya and Ando, 1985), and the anatrepsis is completed when the formation of segmental appendages occurs in the lacewing *Chrysopa perla* (Bock, 1939). The early completion of anatrepsis is a remarkable embryological feature of Raphidioidea. Early anatrepsis has also been reported in other holometabolans such as some groups of Mecoptera (Suzuki, 1990) and Lepidoptera (Kobayashi and Ando, 1988).

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Fig. 2 Eggs of *Inocellia japonica*. A. An egg. B. SEM of an enlargement of the micropylar projection. Micropyles (arrowheads) open around the base of the projection. MpP: micropylar projection. Scales = A: 200 μm ; B: 10 μm .

Figs. 3–6 *Inocellia japonica* eggs in successive stages of embryonic development I. DAPI staining. See the text.

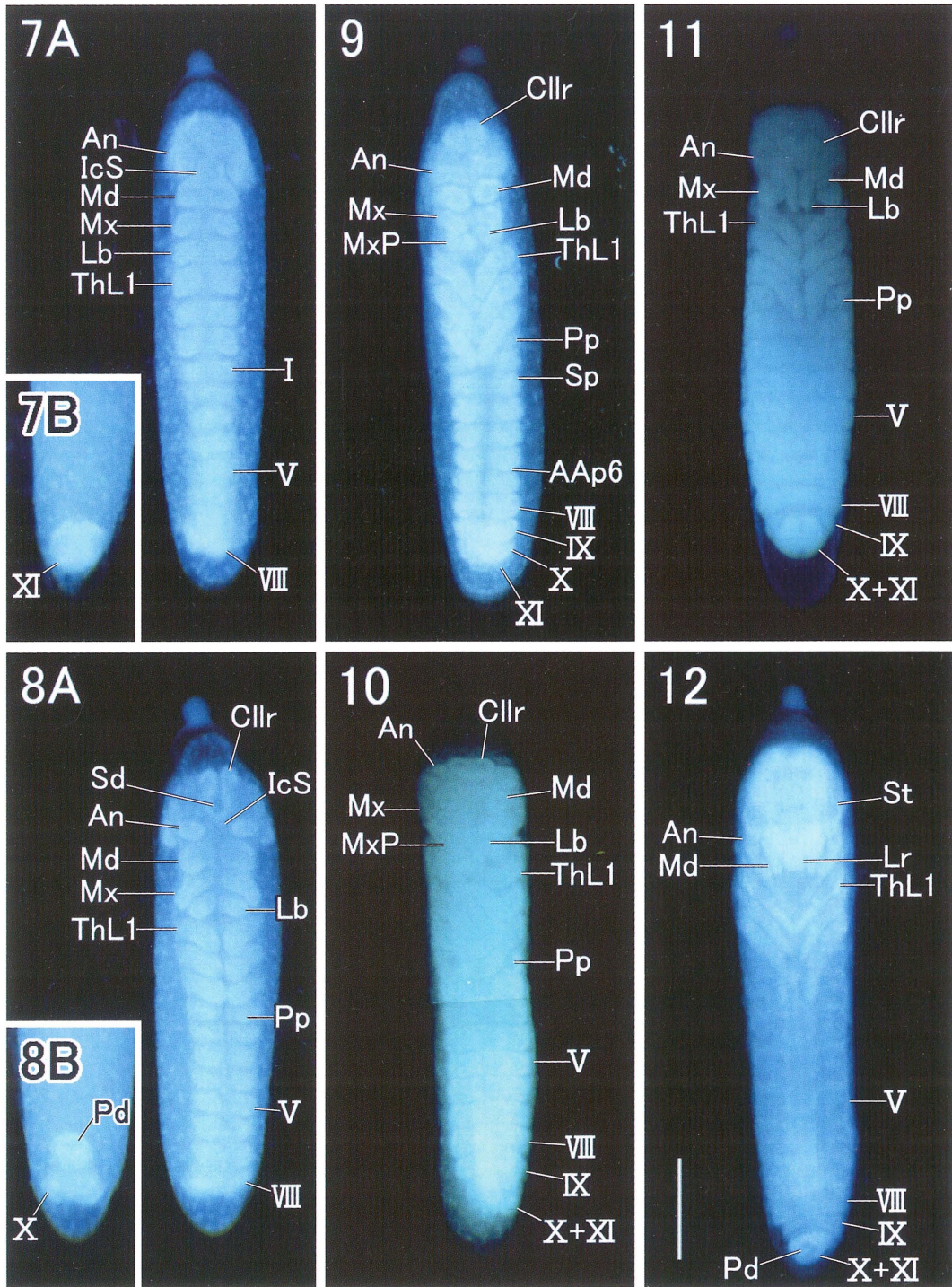
Fig. 3 Early Stage 2

Fig. 4 Late Stage 2

Fig. 5 Lateral view of early Stage 3

Fig. 6 Ventral view of late Stage 3

Bd: blastoderm, CBD: condensed blastoderm, EA: embryonic area, Ec: ectoderm, EeA: extra-embryonic area, PC: pole cell, PG: primitive groove, SYC: secondary yolk cell. Scales = 200 μm .



Figures 7-12

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Figs. 7–12 *Inocellia japonica* eggs in successive stages of embryonic development II. DAPI staining. See the text.

Fig. 7 Ventral (A) and dorsal (B) views of Stage 4.

Fig. 8 Ventral (A) and dorsal (B) views of Stage 5.

Fig. 9 Stage 6.

Fig. 10 Stage 7.

Fig. 11 Stage 8.

Fig. 12 Stage 9.

AAp6: sixth abdominal appendage, An: antenna, Cllr: clypeolabrum, IcS: intercalary segment, Lb: labium, Lr: labrum, Md: mandible, Mx: maxilla, MxP: maxillary palp, Pd: proctodaeum, Pp: pleuropodium, Sd: stomodaeum, Sp: spiracle, St: stemma, ThL1: prothoracic leg, I–XI: first to eleventh abdominal segments. Scale = 200 μ m.