Embryogenesis of the Mayfly *Ephemera japonica* McLachlan (Insecta: Ephemeroptera, Ephemeridae), With Special Reference to Abdominal Formation

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ABSTRACT   Embryogenesis of the mayfly *Ephemera japonica* is described, with special reference to the abdominal morphogenesis. Cleavage is of the typical superficial type. The germ disc is formed by the cell concentration of the embryonic area broadly defined at the posterior half of the blastoderm. The embryo undergoes embryogenesis of the typical short germ type. Blastokinesis in which the extensive and deep invagination of the embryo is involved is similar to those of odonatans and plecopterans. In the longest embryo stage, the abdomen is folded and is divided into four regions (regions I–IV, from anterior to posterior). All the first to eleven segments are derived from regions I and II. Regions III and IV fuse together to form the proctodaeum. This manner of abdominal formation may be regarded as basic in pterygote insects, because a similar manner is found in another palaeopteran group, the odonatans. The caudal filament has been interpreted as the elongation of the eleventh abdominal tergum, but it is now revealed that the caudal filament originates from the posterior extremity of region IV and that its origin is away from the eleventh segment, with regions III and IV or the proctodaeum interposing between. Thus, it is concluded that the caudal filament should be correlated not to the eleventh abdominal segment but to the telson. J. Morphol. *234*:97–107, 1997.   © 1997 Wiley-Liss, Inc.

The Ephemeroptera is an important group in attempting to discuss the phylogeny of pterygote basal clades, which remains controversial (cf. Hennig, '69; Kristensen, '75; Boudreaux, '79), for the Ephemeroptera is widely accepted as one of the pterygote representatives closest to early pterygote ancestors. The Ephemeroptera is also important for understanding the ground plan of the Pterygota as well as for speculation on hexapod evolution.

For phylogenetic discussion, the comparative embryological approach is one of the most promising methods. The embryogenesis of Ephemeroptera has been studied by Joly (1876), Heymons (1896a,b,c), Murphy ('22), Ando and Kawana ('56), Wolf ('60), Bohle ('69), and Tsui and Peters ('74), but details of the ephemeropteran embryology still remain fragmented. We have started a comparative embryological study of the Ephemeroptera using *Ephemera japonica.*

In the first part of our studies, we outline the embryogenesis of the species, with special reference to abdominal morphogenesis.

MATERIALS AND METHODS

The mature females of *Ephemera japonica* McLachlan (suborder Schistonota, family Ephemeridae) were collected at the Kakuma River and at a branch of Karasawa River, Sanada, Nagano Prefecture, Central Japan, in July of 1993 to 1996, and eggs were obtained in the laboratory. The eggs were incubated in water at room temperature ($20 \pm 2°C$). They were fixed with alcoholic Bouin's fluid (saturated alcoholic solution of

picric acid:formalin:acetic acid = 15:5:1) at room temperature for 24 h. Embryos were dissected in Ephrussi-Beadle solution (7.5 g NaCl + 0.35 g KCl + 0.21 g CaCl$_2$/1,000 ml distilled water) with fine forceps and then fixed. The fixed eggs and embryos were stored in 70% ethyl alcohol.

The eggs or embryos were processed into methacrylate resin Technovit 7100 (Kulzer) or water miscible epoxy resin Quetol 651 (Nisshin EM) sections of 1–2 μm thickness, in accordance with Machida et al. ('94a,b). Sections were stained with Delafield's hematoxylin and eosin or, with Schiff's reagent (nucleal or PAS reaction), Mayer's acid hemalum and fast green FCF.

For scanning electron microscopy (SEM), the fixed embryos were sonicated for a few seconds with an ultrasonic cleaner, dehydrated in a graded ethyl alcohol series, and then transferred to acetone. The embryos were dried in a critical-point drier, coated with gold, and observed under a JSM-T200 scanning electron microscope (JEOL, Tokyo).

RESULTS

The egg period of *Ephemera japonica* ranges from 15–17 days at room temperature (20 ± 2°C). Herein we describe the embryonic development, dividing it into 13 stages, with special reference to abdominal development. The most important stages for the abdominal morphogenesis are stages 7–10.

Stage 1: Egg cleavage (Fig. 1A)

Egg cleavage is of the typical superficial type. The first five cleavages are synchronized, each cleavage occurring at intervals of approximately 9 h.

Stage 2: Blastoderm formation (Fig. 1B)

Cleavage nuclei arrive at the egg periphery after eight cleavages, and the syncytial blastoderm (blastema) is formed. Soon the cell membrane appears between the blastoderm cells, and the blastoderm (blastoderm s. str.) is completed.

Stage 3: Germ disc formation (Fig. 1C)

Even in the newly formed blastoderm, the posterior half embryonic and anterior half extraembryonic areas are distinguishable (Fig. 1B). The posterior cells concentrate at the posterior pole of the egg to form the germ

disc. The anterior cells become more flattened and form the future serosa.

Stage 4: Pear-shaped embryo (Fig. 1D,D′)

The germ band starts to elongate backwards. It assumes a pear shape, and the anterior broad protocephalon and posterior protocorm differentiate.

Stage 5: Start of invagination of germ band (Anatrepsis I) (Fig. 1E,E′)

The germ band further elongates, and its caudal end starts to proceed into the yolk. At the same time, the amnion starts to be produced from the embryonic margin, resulting in the start of the amnioserosal fold formation. This amnioserosal fold is derived mainly from the posterior area, the anterior amnioserosal fold being not well developed.

Stage 6: S-shaped embryo (Anatrepsis II) (Fig. 1F,F′)

With active cell proliferation, the embryo further elongates and deeply invaginates into the yolk. The caudal end of the embryo reaches the middle of the egg long axis, and the embryo acquires an S-shape. At this stage, the posterior amnioserosal fold extends anteriorly to the cephalic level, and anatrepsis is completed (Fig. 2). The ventral side of the embryo is completely covered by the amnioserosal fold or the amnion. The inner layer or mesoderm differentiates all over the dorsal side of the embryo (cf. Fig. 3).

Stage 7: Longest embryo (Fig. 1G)

The embryo further elongates, with its posterior half bending three times, and it

Fig. 1. *Ephemera japonica.* Successive stages of embryonic development. Lateral views. I–IV, abdominal regions I–IV; AbS4, 10, 11, fourth, tenth, and eleventh abdominal segments, respectively; Am, amnion; An, antenna; BdC, blastoderm cell; CE, compound eye; Ce, cercus; CF, caudal filament; CN, cleavage nucleus; ET, egg tooth; GD, germ disc; HL, head lobe; Lb, labium; Md, mandibile; Mx, maxilla; Oc, ocellus; Pc, protocephalon; Po, protocorm; PsAm, presumptive amnion; SDO, secondary dorsal organ; Se, serosa; TL1, proleg; Y, yolk. Bar = 50 μm. **A:** Stage 1 (egg cleavage). **B:** Stage 2 (blastoderm formation). **C:** Stage 3 (germ disc formation). **D,D′:** Early (D) and late (D′) stage 4 (pear-shaped embryo). **E,E′:** Early (E) and late (E′) stage 5 (start of invagination of germ band). **F,F′:** Early (F) and late (F′) stage 6 (S-shaped embryo). **G:** Stage 7 (longest embryo). **H:** Stage 8 (segmentation of embryo). **I:** Stage 9 (proctodaeum formation). **J:** Stage 10 (revolution). **K:** Stage 11 (postrevolution I). **L:** Stage 12 (postrevolution II). **M:** Stage 13 (postrevolution III).
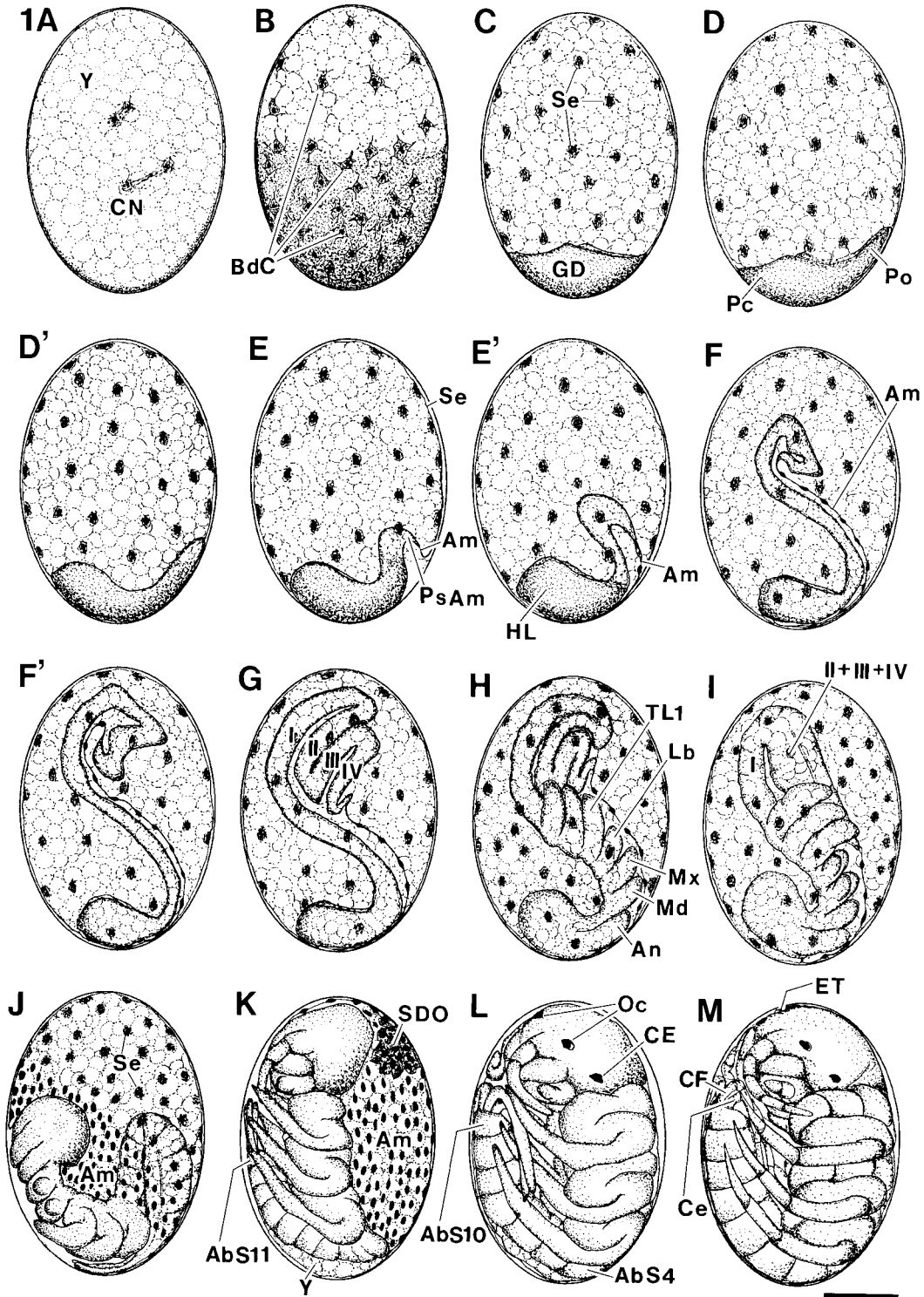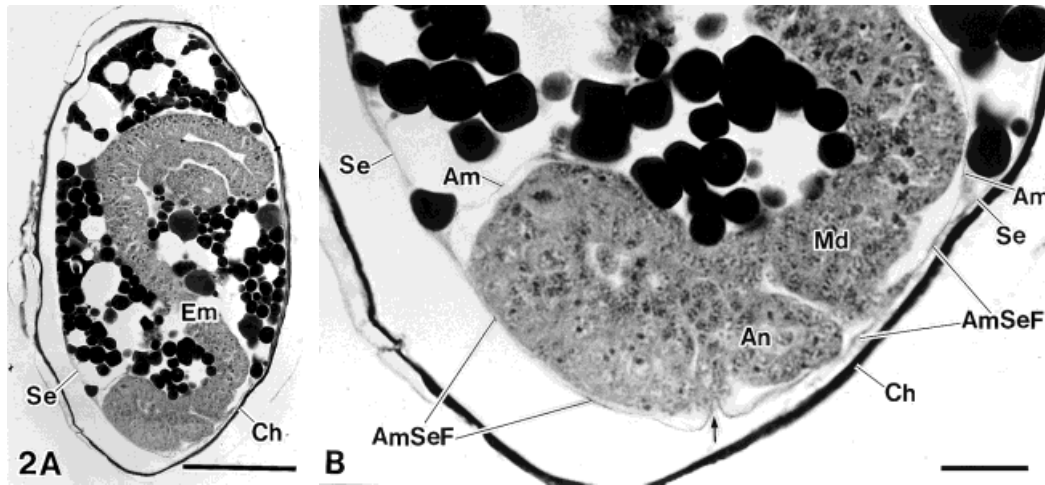
Figure 1

Fig. 2. *Ephemera japonica.* Am, amnion; AmSeF, amnioserosal fold; An, antenna; Ch, chorion; Em, embryo; Md, mandible; Se, serosa. **A:** Sagittal sections of egg, at late stage 6, in which anatrepsis is just completed. Bar = 50 μm. **B:** Enlargement. The amnioserosal folds fuse with each of those beneath the embryo at the level of labrum (arrow), and anatrepsis is completed. Bar = 10 μm.

acquires its maximum length, reaching near the anterior egg pole. With the bendings, the posterior half of embryo or the future abdomen is folded and divided into four regions (regions I–IV from the anterior to posterior [Fig. 3A]). In cross-section, regions I and II and regions III and IV are respectively connected to each other by the amnion (cf. Fig. 3B), and all of the regions are dorsally lined with the inner layer (Fig. 3B).

Stage 8: Segmentation of embryo (Fig. 1H)

The segmentation of embryo commences. It proceeds from the head to the rear, and simultaneously appendage rudiments appear in the cephalic and thoracic segments.

In regions I and II of the abdomen, now divided into four regions, the first to fifth and the sixth to eleventh abdominal segments, respectively, develop (Figs. 5A, 9A). Late in this stage, neuroblasts and neuropiles can be clearly observed in all the first to eleventh abdominal segments (Fig. 5A,B; cf. Fig. 6A,B). In contrast, no attributes of a segmental nature can be found in regions III and IV.

Stage 9: Proctodaeum formation (Fig. 1I)

The appendages of the head and thorax are enlarged and segmented. Regions III and IV of the abdomen fuse with each other dorsally to form the proctodaeum (Figs. 3B, 4A, 9B,B′). Then the proctodaeum itself is

soon enclosed by the definitive dorsal closure of region II (Figs. 4A, 9B,B′,C).

The caudal filament develops at the apex of region IV (Figs. 4B, 7A,B, 8, 9). A pair of cerci develops at the eleventh abdominal segment, which is the extremity of region II. With the progression of definitive dorsal closure, the cerci move from the original ventrolateral to the dorsolateral position and reach their definitive position at the same level as the caudal filament (Figs. 7, 9B vs. Figs. 8, 9C).
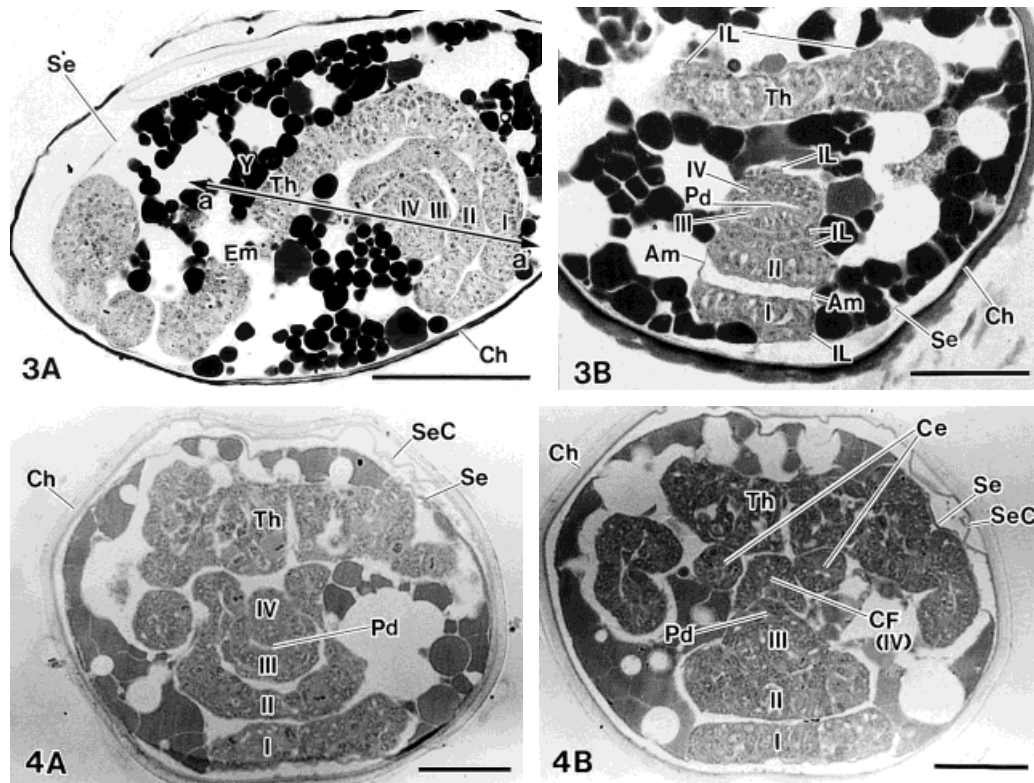
Stage 10: Revolution (Katatrepsis) (Fig. 1J)

The amnioserosal fold tears near the labrum, and the embryo appears on the egg surface. The embryo, which is slightly shortened temporarily, moves along the ventral surface of the egg towards the anterior pole, with its head to the traveling direction, and the antero-posterior axis of the embryo reverses.

Stage 11: Postrevolution I (Fig. 1K)

The serosal cells are condensed at the antero-dorsal part of the egg to form the secondary dorsal organ just posteriorly to the head. With the progressive condensation and withdrawal of serosal cells, the amnion replaces the serosa and finally spreads over the dorsal yolk as a provisional dorsal closure.

Figs. 3, 4. *Ephemera japonica.* Sections of eggs at stages 7 and 9.

Fig. 3. Eggs of stage 7. I–IV, abdominal regions I–IV; Am, amnion; Ch, chorion; Em, embryo; IL, inner layer; Pd, proctodaeum; Se, serosa; Th, thorax; Y, yolk. **A:** Sagittal section. Embryo acquires its maximum length with its posterior half bending three times. With bending, the posterior half of the embryo or the future abdomen is folded and divided into four regions (regions I–IV). Bar = 50 μm. **B:** Cross-section approximately through a–a′ level in A. In this stage, regions I and II and regions III and IV are, respectively, connected with each other by the amnion, but between region III and IV the amniotic communications are not found, because

these regions on this plane have already fused with each other. Regions I–IV are found to be dorsally lined with the inner layer. Bar = 20 μm.

Fig. 4. Eggs of stage 9. I–IV, abdominal regions I–IV; Ce, cercus; CF, caudal filament; Ch, chorion; Pd, proctodaeum; Se, serosa; SeC, serosal cuticle; Th, thorax. Bars = 25 μm. **A:** Cross-section at the eleventh abdominal segment. Regions III and IV of the abdomen or the proctodaeum is enclosed by region II. **B:** Cross-section at the base of the caudal filament and cerci. From A and B, it is clear that the caudal filament develops at the apex of region IV and that a pair of cerci develops at the eleventh abdominal segment, which is the extremity of region II.

Stage 12: Postrevolution II (Fig. 1L)

The secondary dorsal organ which formed at the previous stage sinks into the yolk and disappears. The appendages of the head and thorax further develop. The definitive dorsal closure further proceeds anteriorwards. A pair of cerci and the caudal filament elongate and become segmented. Late in this stage, compound eyes and three ocelli can be clearly observed.

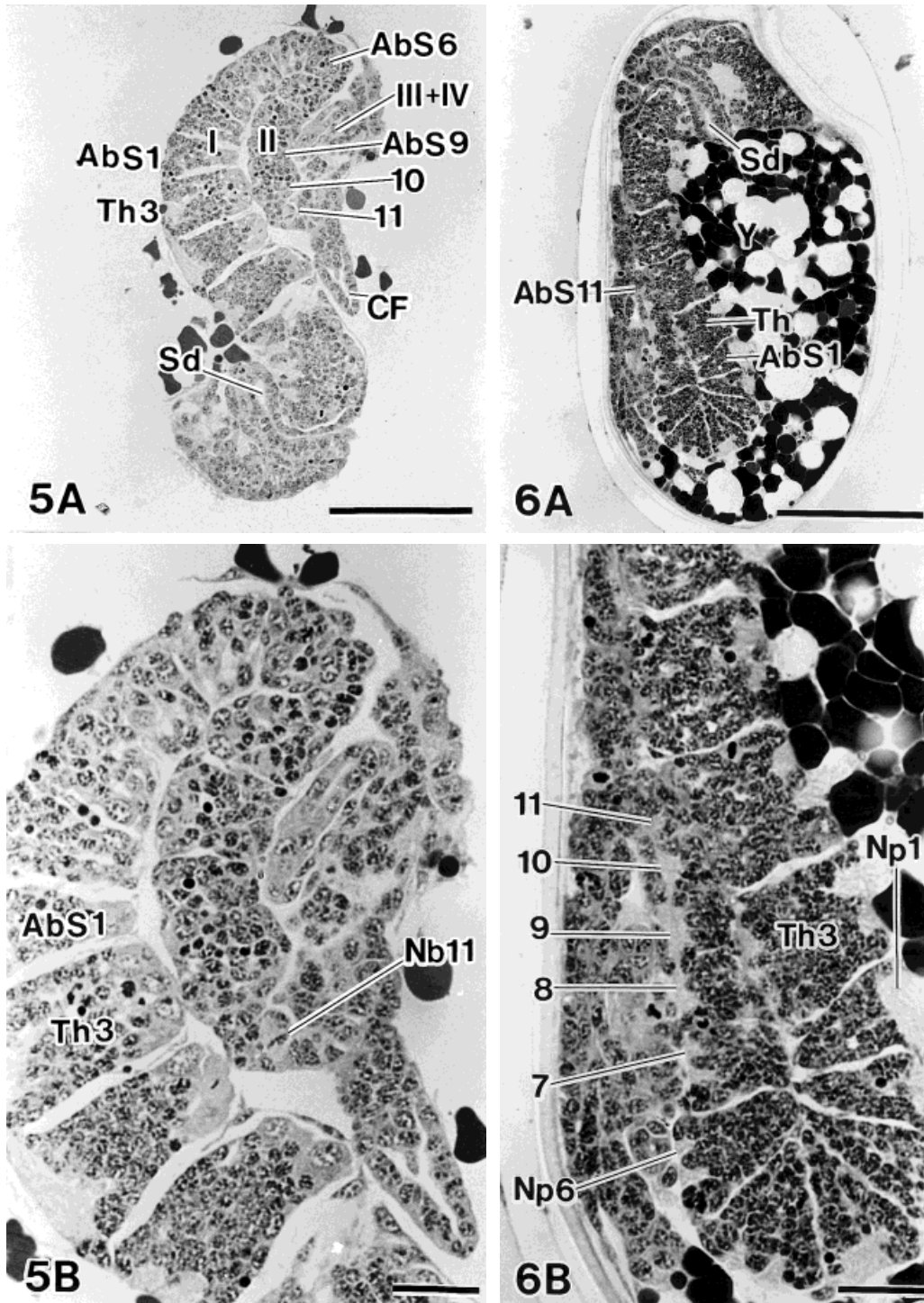### Stage 13: Postrevolution III (Fig. 1M)

The definitive dorsal closure is completed, and the embryo acquires its definitive form.

The larval cuticle is secreted, and a sclerotized egg tooth is visible on the frons.

DISCUSSION
General aspects of embryogenesis

The germ disc of *Ephemera japonica* is formed with a concentration of cells at the embryonic area that is broadly defined at the posterior half of the blastoderm. A similar manner of germ disc formation is observed in other ephemeropterans (i.e., *Ephemera strigata* [Ando and Kawana, '56], *Baetis rhodani* and *B. vernus* [Bohle, '69]),

Figures 5–6

and this manner of germ disc formation may be regarded as an important characteristic of ephemeropteran embryogenesis.

The embryo of *Ephemera japonica* can be categorized as the typical short germ type (cf. Krause, '39), characterized by the sequential proliferation of segments from the anterior to posterior. Because the short germ type dominates in primitive pterygotes (e.g., Odonata [Ando, '62] Plecoptera [Kishimoto and Ando, '85]) as well as in ectognathan apterygotes (cf. Sander, '84), this may be regarded as an ancestral feature. As a result of anatrepsis and elongation of the germ band, the embryo of *Ephemera japonica* acquires an S-shape, as observed in other primitive pterygotes such as an odonatan *Epiophebia superstes* (Ando, '62) and a plecopteran *Kamimuria tibialis* (Kishimoto and Ando, '85), and this type of blastokinesis may be considered to be plesiomorphic within the pterygotes.

It has been confirmed that in *Ephemera japonica* the serosal cuticle is not secreted until the completion of anatrepsis—that is, until the embryo is ventrally covered by the amnioserosal fold and the entire egg surface is occupied by serosa. This may be favorable to the idea of Machida et al. ('94a) concerning the functional role of the amnioserosal fold. They deduced that the amnioserosal fold should have been acquired during ectognathan evolution in order to secrete the serosal cuticle beneath the embryo that had lost this ability.

Proctodaeum formation

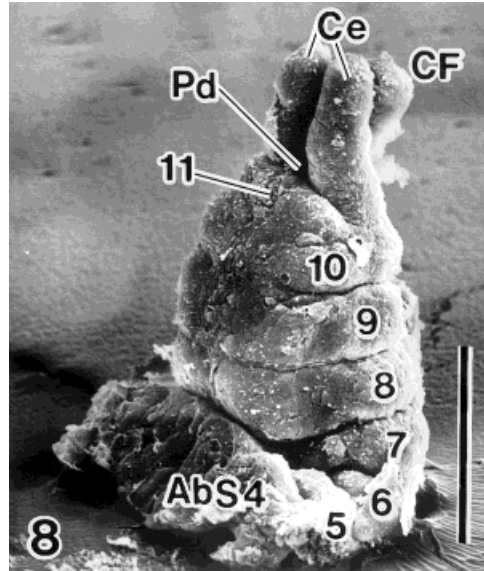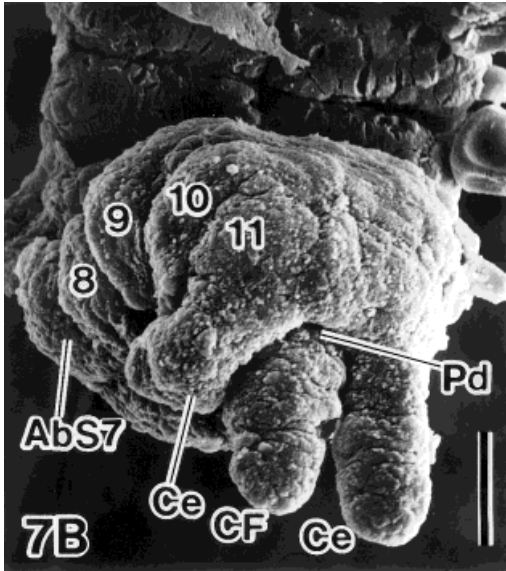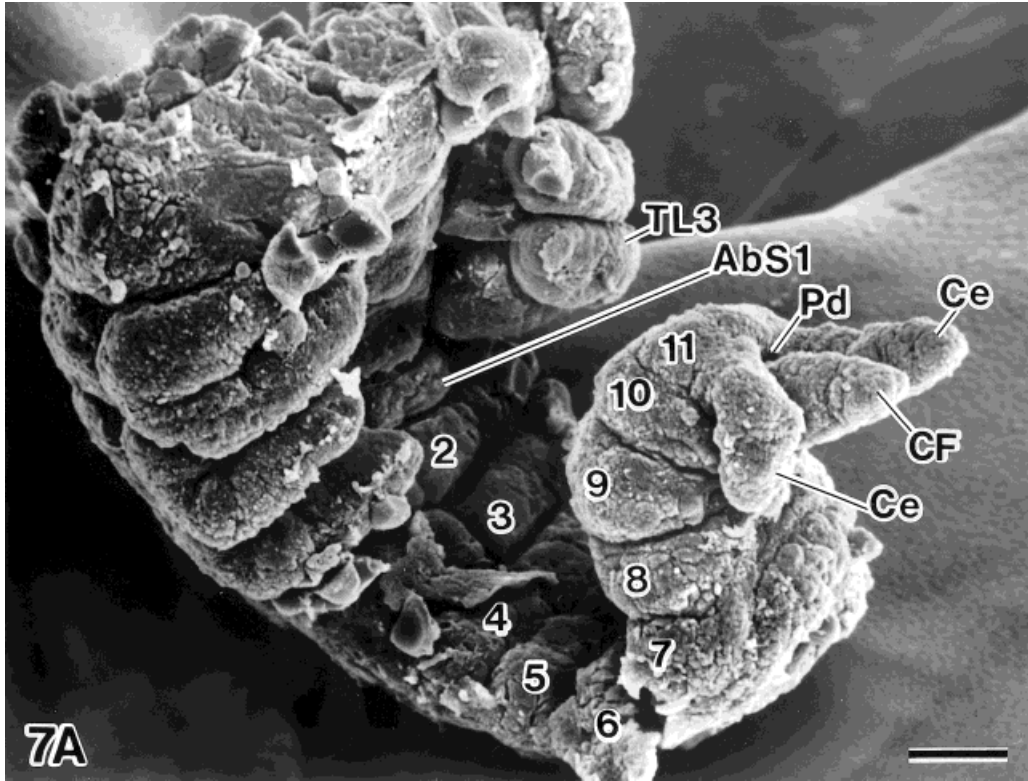In the longest embryo stage (stage 7), the abdomen of *Ephemera japonica* is folded and

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Figs. 5, 6. *Ephemera japonica.* Sagittal sections of the embryo at stage 8 and egg at stage 11.

Fig. 5. I–IV, abdominal regions I–IV; AbS1, 6, 9–11, first, sixth, and ninth to eleventh abdominal segments; CF, caudal filament; Nb11, neuroblast of eleventh abdominal segment; Sd, stomodaeum; Th3, metathorax. **A:** Embryo just before katatrepsis at stage 8. Bar = 50 µm. **B:** Enlargement of abdomen. A neuroblast is clearly observed in the eleventh abdominal segment. Bar = 10 µm.

Fig. 6. AbS1 and 11, first and eleventh abdominal segments; Np1, 6–11, neuropiles in the first and sixth to eleventh abdominal segments; Sd, stomodaeum; Th, thorax; Th3, metathorax; Y, yolk. **A:** Egg at stage 11. Bar = 50 µm. **B:** Enlargement of abdomen. Neuropiles are clearly differentiated in the first to eleventh abdominal segments. Bar = 10 µm.

divided into four regions, I–IV. Regions III and IV fuse together to form the proctodaeum; the ventral and dorsal walls, respectively, originate from regions III and IV. A similar manner of proctodaeum formation is found in another palaeopteran group, the Odonata (Ando, '62), and this type of proctodaeum formation, in which the proctodaeum is formed by the fusion of two belt-like rudiments, may be regarded as one of the most basic in Pterygota.

The proctodaeum formations in neopteran orders may be categorized as this type, such as the hemipterans *Pyrrhocoris apterus* (Seidel, '24) and *Oncopeltus fasciatus* (Butt, '49), the homopteran *Pyrilla perpusilla* (Sander, '56), the mecopteran *Panorpa pryeri* (Suzuki and Ando, '81), the trichopteran *Stenopsyche griseipennis* (Miyakawa, '75), and the lower lepidopterans *Endoclita signifer* (Kobayashi et al., '81) and *Neomicropteryx nipponensis* (Kobayashi and Ando, '88). In these insects, region IV has been regarded as amniotic in origin, because of its lack of inner layer, and their proctodaeum has been recognized to be dual in origin (i.e., originating from the dorsal wall of the amnion and the ventral wall of the embryo proper) (Seidel ('24) observed that both the dorsal and ventral walls of *P. apterus* proctodaeum lack the inner layer, and he concluded the whole proctodaeum of this insect to be amniotic in origin) (cf. papers mentioned above). In light of this, it may also be said that region IV, namely the whole proctodaeum, of the Palaeoptera (Ephemeroptera (herein) Odonata [Ando, '62]) is embryonic proper in origin, because the inner layer is found to be segregated also on region IV, namely on the entire proctodaeum.

Accordingly, we may recognize the difference in proctodaeum formation, concerning the differentiation of the inner layer, between the palaeopterans and neopterans. However, we suggest that the difference is not so essential as to specifically designate the partially different origin of the proctodaeum. We believe that the difference can be explained merely by the term *heterochrony,* concerning the timing of the segregation of the inner layer and the morphogenesis of the proctodaeum, or by the phrase "substitution in the developmental process of Matsuda ('76)," namely the evolutional alteration of the manner of mesodermal supply to the wall of the proctodaeum. Since we cannot find any new bases for the amniotic nature

Figs. 7, 8. *Ephemera japonica.* SEM micrographs of abdomens at stage 9.

Fig. 7. AbS1–11, first to eleventh abdominal segments; Ce, cercus; CF, caudal filament; Pd, proctodaeum; TL3, metaleg. Bars = 10 μm. **A:** Ventro-lateral view of the abdomen. **B:** Enlargement from a different angle. It is clear that the origins of cerci and the caudal filament are different.

Fig. 8. Ventro-lateral view of the abdomen, more developed than that in Fig. 7. The cerci move from their original ventro-lateral to the dorso-lateral position, and they reach their definitive position at the same level as the caudal filament. AbS4–11, fourth to eleventh abdominal segments; Ce, cercus; CF, caudal filament; Pd, proctodaeum. Bar = 30 μm.
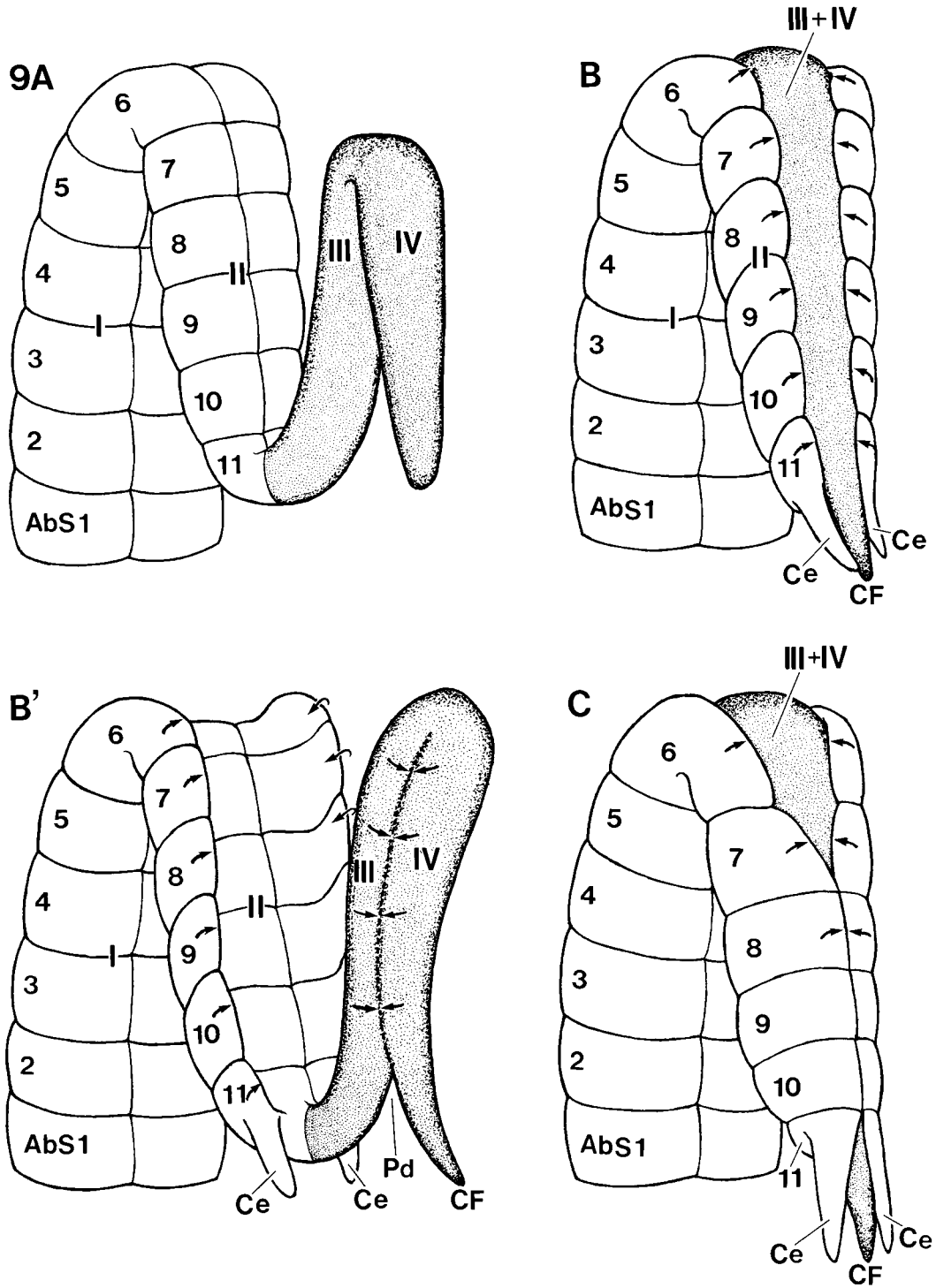
Fig. 9. *Ephemera japonica.* Diagrammatic representation of abdominal development. I–IV, abdominal regions I–IV; AbS1–11, first to eleventh abdominal segments; Ce, cercus; CF, caudal filament; Pd, proctodaeum. **A:** Stage 8. **B,B′:** Early stage 9. In B′, regions III and IV are pulled apart from region II to show the proctodaeum in formation. **C:** Late stage 9. The proctodaeum is enclosed by the definitive dorsal closure of region II.

of region IV in neopterans, it is better to recognize the region to be of the embryo proper in origin as well.

Caudal filament and abdominal formation

Heymons (1896a,b,c) deduced that the caudal filament is the elongation of the eleventh abdominal tergum. His idea has been followed by many insect morphologists, such as Crampton ('17, '18), Snodgrass ('35), Brinck ('57), Birket-Smith ('71), and Matsuda ('76), and has remained largely unchallenged.

In our present study, we clarify that the caudal filament of *Ephemera japonica* originates from the posterior extremity of region IV and has its origin considerably away from the eleventh abdominal segment, with regions III and IV or the precursor of proctodaeum interposing (Figs. 7–9). If the caudal filament, as Heymons (1896a,b,c) deduced, is a derivative of the eleventh abdominal segment, we should regard regions III and IV or the proctodaeum as the eleventh abdominal segment as well and suppose an enormously long eleventh abdominal segment. Regarding the derivation of the caudal filament, another interpretation may be more plausible. That is, the caudal filament should not be attributed to the eleventh abdominal segment but should be correlated to the so-called telson that has been thought to be represented in insects only by the proctodaeum and anal lobes (cf. Snodgrass, '35; Matsuda, '76).

Abdominal formation and metamerism of *Ephemera japonica* can be summarized as follows. Its embryonic abdomen is composed of four regions, I–IV. From regions I and II, the first to fifth and the sixth to eleventh abdominal segments originate, respectively. Each of these eleven segments is characterized by a pair of ganglia, and the eleventh is characterized by a pair of cerci as appendages as well. Regions III and IV following the eleventh abdominal segment are devoid of any attributes suggestive of "segments." Regions III and IV develop into the proctodaeum. At the terminal of region IV, the caudal filament forms as a derivative of the telson which may be represented by the proctodaeum and anal lobes.

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