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Early Embryonic Development of the Mayfly *Ephemera japonica* McLachlan (Insecta: Ephemeroptera, Ephemeridae)

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ABSTRACT In the newly laid egg of the mayfly *Ephemera japonica*, an egg nucleus (oocyte nucleus) at metaphase of the first maturation division is in the polar plasm at the mid-ventral side of the egg, and a male pronucleus lies in the periplasm beneath a micropyle situated just opposite the polar plasm or at the mid-dorsal side of egg. The maturation divisions are typical. An extensive and circuitous migration of the male pronucleus is involved in the fertilization process: it first moves anteriad in the periplasm from beneath the micropyle to the anterior pole of the egg and then turns posteriad in the yolk along the egg's long axis to the site of syngamy, near the center of the egg. Cleavage is superficial. The successive eight cleavages, of which the first five are synchronized, result in the formation of the blastoderm, and about ten primary yolk cells remain behind in the yolk. Even in the newly formed blastoderm, the thick embryonic posterior half and the thin extraembryonic anterior half areas are distinguished: the former cells are concentrated at the posterior pole of the egg to form the germ disc, and the latter cells become more flattened, forming serosa. Time-lapse VTR observations reveal a yolk stream that is in accord with the migration of the male pronucleus in time and direction. The yolk stream is also generated in activated unfertilized eggs, and it is probable that the migration of the male pronucleus in association with the fertilization may be directed by the yolk stream. J. Morphol. 238:327–335, 1998.

KEY WORDS: mayfly; Ephemeroptera; Insecta; embryogenesis; maturation; fertilization; yolk stream; cleavage; fusion nucleus; pronuclei

The Ephemeroptera is regarded as one of the representatives closest to early pterygote ancestors. It is an important group in attempting to understand the ground plan of Insecta, and in discussions of the phylogeny of pterygote basal clades, which remains controversial (Hennig, '69; Kristensen, '75; Boudreaux, '79). The comparative embryological approach is one of the most promising methods for phylogenetic analyses. Regarding the embryogenesis of Ephemeroptera, although one can refer to several articles such as Joly (1876), Heymons (1896a,b,c), Murphy ('22), Ando and Kawana ('56), Wolf ('60), Bohle ('69), and Tsui and Peters ('74), the details of ephemeropteran embryology still remain insufficient and fragmented.

For this reason, we have been conducting a comparative embryological study of the Ephemeroptera (Tojo and Machida, '96, '97a,b, '98, '99). As part of this ongoing research, in the present study we describe in detail the early embryonic development of Ephemeroptera, which has been unknown, with special reference to fertilization, using *Ephemera japonica* (Ephemeridae).

MATERIALS AND METHODS

Mated, mature females of *Ephemera japonica* McLachlan (suborder Schistonota, family Ephemeridae) were collected at a branch of the Karasawa River, Sanada, Nagano Prefecture, Central Japan, from July to August of 1996 and 1997, and eggs were obtained in the laboratory. Unfertilized eggs were dissected out of mature, uncopulated

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imagos or subimagos, or ultimate instar nymphs.

The eggs were incubated in water at room temperature (20 ± 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 hours. The fixed eggs were stored in 70% ethyl alcohol. A small opening was made by fine forceps in the chorion of some eggs prior to fixation. The eggs were fixed with Karnovsky's fixative (2% paraformaldehyde + 2.5% glutaraldehyde) buffered at pH 7.2 with HCl-sodium cacodylate at 4°C for 2 hours. They were then stored in the HCl-sodium cacodylate buffer at 4°C.

The fixed eggs were processed into methacrylate resin Technovit 7100 (Kulzer, Wehrheim):styrene = 4:1 or Technovit 8100 (Kulzer) sections of 1–2 µm thickness, in accordance with Machida et al. ('94a, b). Some eggs were immersed in a solution of 70% ethanol:50% ammonium mercaptoacetate = 9:1 for a few hours, prior to the infiltration of resin, to soften the chorion.

Sections were stained with Delafield's hematoxylin and eosin (in some cases supplementarily stained with 0.1% fast green FCF) or with Azan (Domagk's modification). Some newly laid eggs were stained with the DNA-specific fluorescent dye Hoechst 33342 (Calbiochem, La Jolla, CA) after a small opening was made in the chorion.

Some newly laid and some unfertilized living eggs were observed with a time-lapse VTR system (Olympus, Tokyo: CK-2 inverted microscope; Victor, Tokyo: SR-9070 video tape recorder; and Kenis, Osaka: QB CCD camera).

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

OBSERVATIONS

Maturation and fertilization

The newly laid egg (oocyte s. str.) of *Ephemera japonica* is ellipsoidal, about 200 µm by 100 µm (Fig. 1A) . The egg's long axis corresponds to the anterior-posterior axis, from the observation of developed eggs. At the time of oviposition, the egg nucleus (oocyte nucleus s. str.), which is in the polar plasm (cytoplasmic island) that proves to be located in the mid-ventral side of the egg from the observation of later eggs, is at the metaphase of the first maturation (meiotic) division. Its spindle axis is vertical to the egg surface (Figs. 2, 3A,C, 4; also see Fig. 14A) . The first maturation division has already started at the ultimate instar nymphal stage, but it is arrested at metaphase, so that the division is equational. One micro-

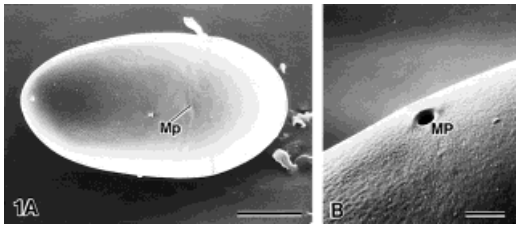


Fig. 1. *Ephemera japonica*. SEM micrographs of an egg. Mp, micropyle. **A:** Egg with adhesive layer. Bar = 50 µm. **B:** Enlargement of micropyle. Bar = 2 µm.

Figs. 2–6. *Ephemera japonica*. Early embryonic development I.

Fig. 2. Newly laid egg. Lateral view (anterior to the top, ventral to the left). The egg was stained with Hoechst 33342 and observed through a green filter (UV excitation, fluorescence microscopy). The egg nucleus (oocyte nucleus s. str.) and male pronucleus can be seen at the ventral (arrowhead) and opposite or dorsal (arrow) sides of the egg, respectively. The vague fluorescence at the anterior egg pole was artificially caused by pricking the chorion. Bar = 50 µm.

Fig. 3. Longitudinal sections of the egg shown in Figure 2. Ch, chorion; Chr, chromatin of egg nucleus (oocyte nucleus); MPn, male pronucleus; PP, polar plasm (cytoplasmic island); Y, yolk. **A:** Section with polar plasm. Bar = 50 µm. **B:** Section with male pronucleus. A and B are 10 µm apart. Bar = 50 µm. **C:** Enlargement of A. Bar = 5 µm. **D:** Enlargement of B. Bar = 5 µm.

Fig. 4. Longitudinal section of the polar plasm of a newly laid egg (metaphase of the first maturation division). Ch, chorion; Chr, chromatin of egg nucleus; PP, polar plasm (cytoplasmic island); Y, yolk. Bar = 5 µm.

Fig. 5. Longitudinal sections of egg, at the telophase of the second maturation division, 1.5 hours after oviposition. Ch, chorion; Chr, chromatin of egg nucleus; PB_1, first polar body; PB_2, second polar body; PP, polar plasm; Y, yolk. Bars = 5 µm. **A:** Section through first polar body and egg nucleus. **B:** Section through second polar body. A and B are 4 µm apart.

Fig. 6. Longitudinal section of egg 1.5 hours after oviposition. The second maturation division finishes and the female pronucleus starts its migration toward the center of the egg. Ch, chorion; FPn, female pronucleus; PP, polar plasm; Y, yolk. Bar = 5 µm.

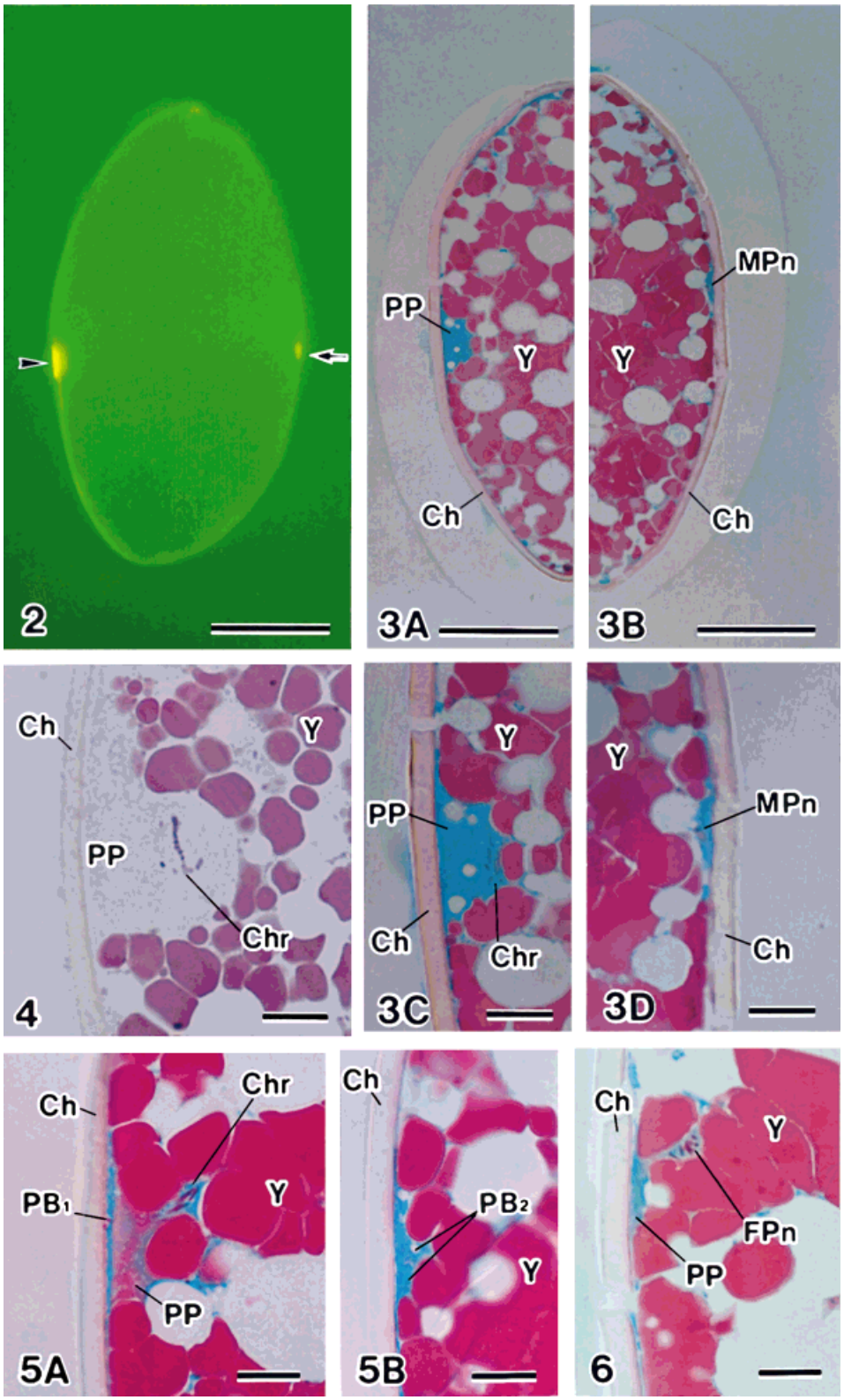

2
3A
PP
Y
Ch
3B
MPn
Y
Ch
4
Ch
Y
PP
Chr
3C
Y
PP
Ch
Chr
3D
Y
MPn
Ch
5A
Ch
Chr
Y
PB1
PP
5B
Ch
PB2
Y
6
Ch
Y
FPn
PP

Figure 2–6

pyle is situated on the equator (Fig. 1), opposite to the polar plasm, i.e., the mid-dorsal side of the egg, and a male pronucleus, derived from a spermatozoon, is found just beneath the micropyle (Figs. 2, 3B,D; also see Fig. 14A).

At 0.5–1 hour after oviposition, the first maturation division finishes, the first polar body is formed, and moves just beneath the chorion in the polar plasm (see Fig. 14B; cf. Fig. 5A). The first polar body begins to degenerate at this point, and we could not determine its fate, i.e., whether it undergoes the following division.

At 1.5 hours after oviposition, the second maturation division occurs, producing the female pronucleus, and the second polar body is given off toward the outer periphery of the polar plasm (Fig. 5B; also see Fig.14B,C). The female pronucleus soon starts to migrate into the yolk toward the center of the egg, accompanied by part of the polar plasm (Fig. 6; also see Fig. 14C). During the second maturation division, the male pronucleus starts to migrate toward the anterior pole in the periplasm (see Fig. 14B,C; cf. Fig. 7) .

At 2–3 hours after oviposition, the female and male pronuclei arrive at the center and the anterior pole of egg, respectively (Figs. 7, 8, 14D) . In a living egg, the male pronucleus accompanied by cytoplasm is recognized as a depression in the yolk at the anterior pole of the egg (Fig. 8A). On arriving there, the male pronucleus changes its direction inwards into the yolk and proceeds along the long axis of the egg to approach the female pronucleus, accompanied by surrounding cytoplasm (Figs. 9, 14E,F). As a result, the anterior yolk depression gradually becomes shallow. The surrounding cytoplasm of the male pronucleus is partially left behind on the path that the male pronucleus has traced from the anterior pole to the center of the egg (Figs. 9, 14F).

Thus, the male pronucleus migrates and approaches the female pronucleus. The female pronucleus exhibits an approaching movement, although slight, toward the male pronucleus just before their conjugation. At 5–6 hours after oviposition, the male and female pronuclei conjugate with each other near the center of the egg and fertilization is completed (Figs. 10, 14G).

Time-lapse VTR reveals not only the migration of pronuclei in the yolk but also a yolk stream that coordinates in time and direction with the migration of the male pronucleus mentioned above. During the prefertilization stage, the yolk stream first moves anteriad at the egg surface and then enters and sinks inward into the yolk from the anterior pole of the egg (Fig. 15A-C) . Fertilization then occurs and a reversal stream involving the whole yolk takes place (Fig. 15D).

Unfertilized egg

In unfertilized eggs of *Ephemera japonica*, which are activated by immersing in water, a yolk depression is also formed at the anterior pole of the egg 2–3 hours after activation (Fig. 16) . Time-lapse VTR shows that in unfertilized eggs, a yolk stream is generated that is similar to that observed in the prefertilization stage of fertilized eggs, although the unfertilized egg yolk stream is slightly less extensive (Fig. 17).

Figs. 7–13. *Ephemera japonica*. Early embryonic development II.

Fig. 7. Longitudinal section of an egg about 2 hours after oviposition. The male pronucleus is migrating toward the anterior pole in the periplasm and the female pronucleus arrives at the center of the egg. Ch, chorion; FPn, female pronucleus; MPn, male pronucleus; Y, yolk. **A:** Anterior part of longitudinal section. Bar = 50 µm. **B:** Enlargement. Bar = 10 µm.

Fig. 8. Anterior region of eggs about 2.5 hours after oviposition. The male pronucleus arrives at the anterior egg pole. Ch, chorion; MPn, male pronucleus; Y, yolk. Bars = 10 µm. **A:** Living egg. The cytoplasm surrounding the male pronucleus is recognized as a depression of yolk at the anterior pole of the egg (arrow). **B:** Sectioned egg.

Fig. 9. Longitudinal section of an egg 3 hours after oviposition. The male pronucleus migrates in the yolk along the egg long axis, approaching the female pronucleus. Ch, chorion; FPn, female pronucleus; MPn, male pronucleus; RCMPn, remnant of the surrounding cytoplasm of the male pronucleus; Y, yolk. Bar = 20 µm.

Fig. 10. Longitudinal section of an egg at fertilization about 5.5 hours after oviposition. Ch, chorion; Sk, synkaryon; Y, yolk. Bar = 20 µm.

Fig. 11. Longitudinal section of an egg at first cleavage division 7 hours after oviposition. Ch, chorion; CN, cleavage nucleus; Y, yolk. Bar = 20 µm.

Fig. 12. Longitudinal section of an egg at the sixth cleavage stage 2 days after oviposition. Ch, chorion; CN, cleavage nucleus; Y, yolk. Bar = 20 µm.

Fig. 13. Longitudinal section of an egg at the cellular blastoderm stage about 3 days after oviposition. BdC, blastodermal cell; Ch, chorion; Y, yolk. Bar = 20 µm.

Cleavage, blastoderm, and germ disc formation

Cleavage of *Ephemera japonica* is of the typical superficial type. About 10 hours after oviposition, the first cleavage takes place at the site of fertilization or at the center of the egg, with its spindle axis oblique to the long axis of the egg (Figs. 11, 14H). The second cleavage occurs vertically to the direction of the first cleavage (Fig. 14I). The following cleavages occur at intervals of approximately 9 hours. The first five cleavages are synchronized in phase and the number of nuclei accords with the 2^n -rule (Fig. 14H–L).

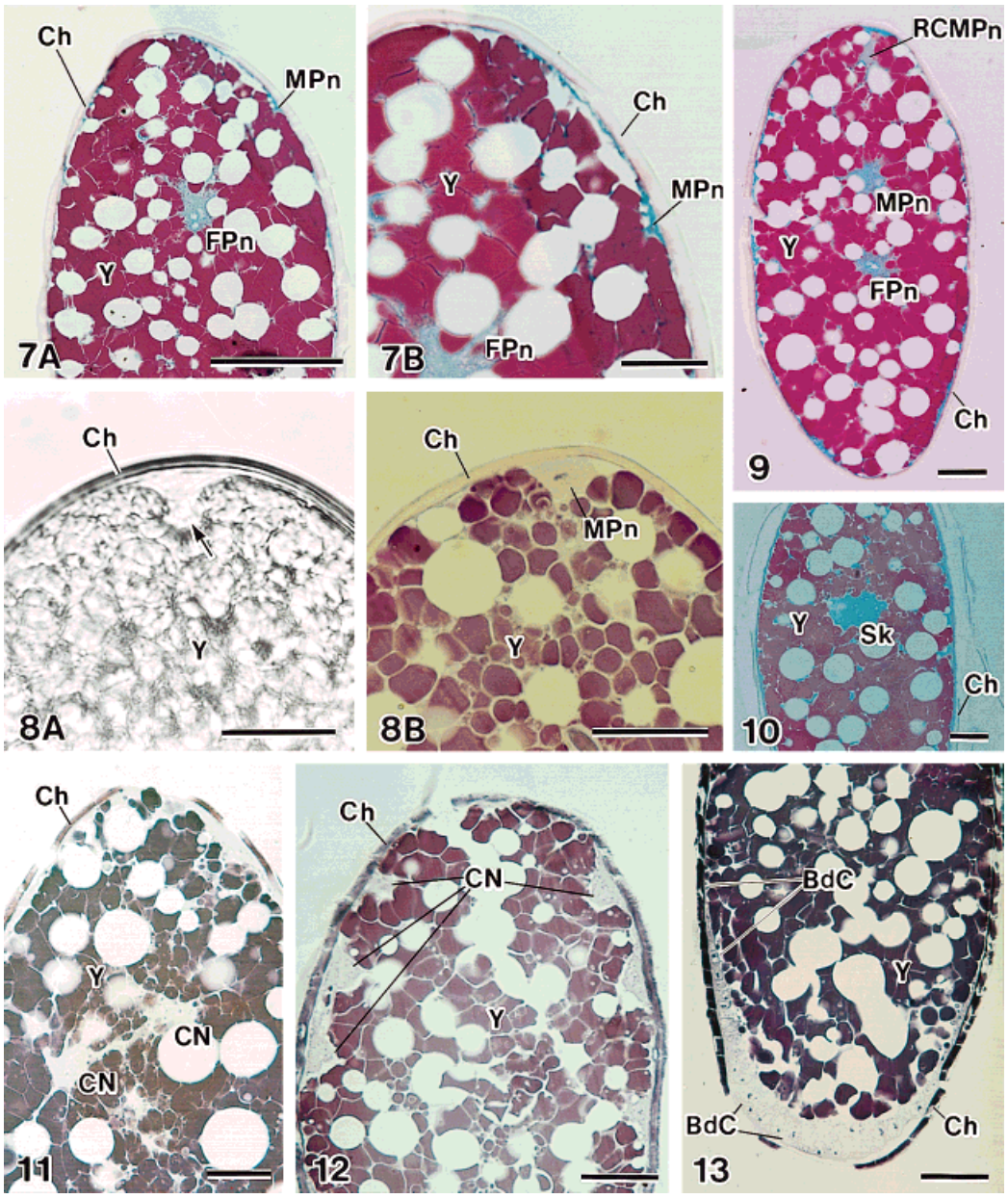


Figure 7–13

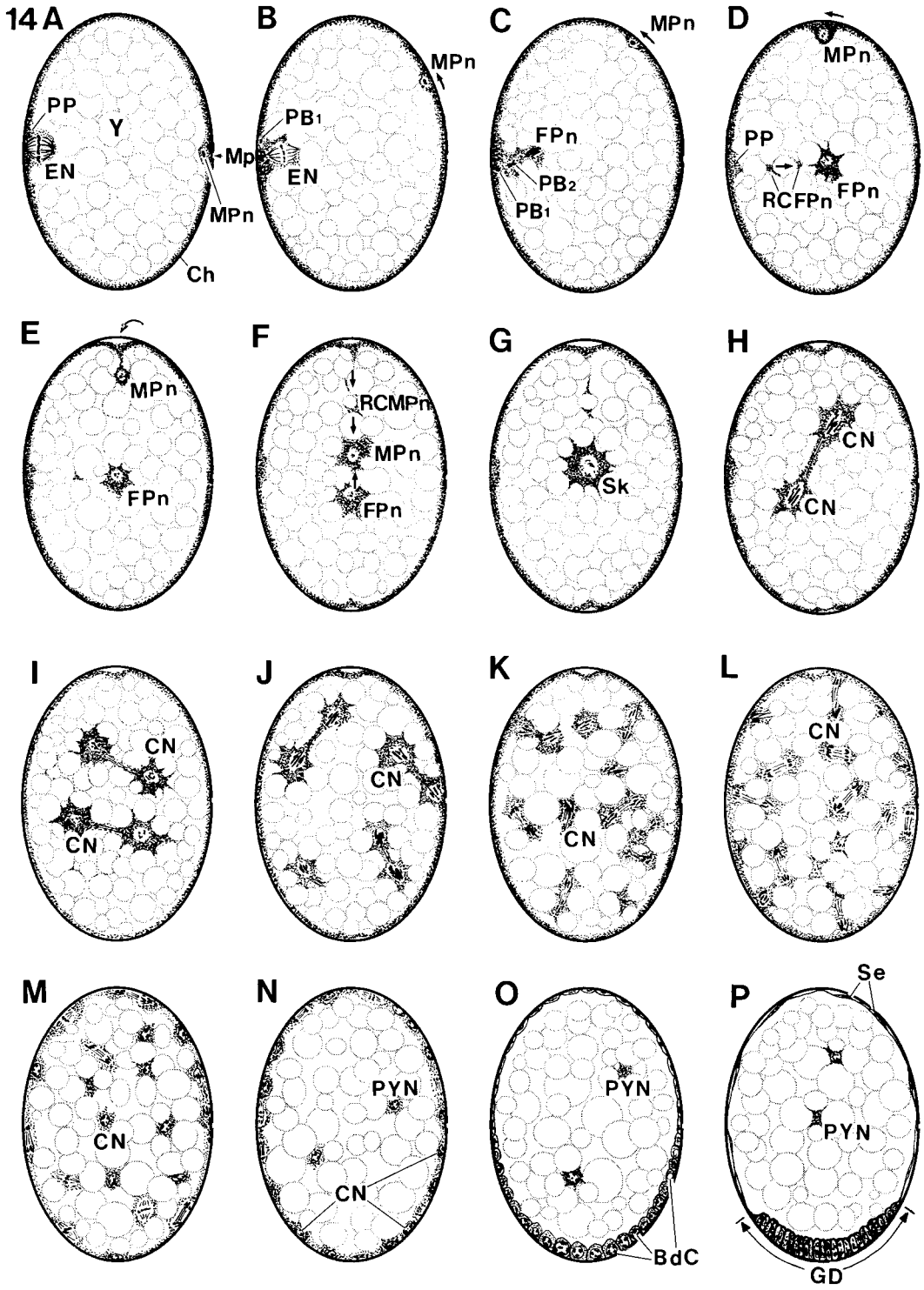


Fig. 14. *Ephemera japonica*. Diagrammatic representation of the developmental process from oviposition to germ disc formation (**A–P**). See text. BdC, blastodermal cell; Ch, chorion; CN, cleavage nucleus; EN, egg nucleus (oocyte nucleus); FPn, female pronucleus; GD, germ disc; Mp, micropyle; MPn, male pronucleus; PB_1, first polar body; PB_2, second polar body; PP, polar plasm (cytoplasmic island); PYN, primary yolk nucleus; RCFPn, remnant of cytoplasm of female pronucleus; RCMPn, remnant of cytoplasm of male pronucleus; Se, serosa; Sk, synkaryon; Y, yolk.

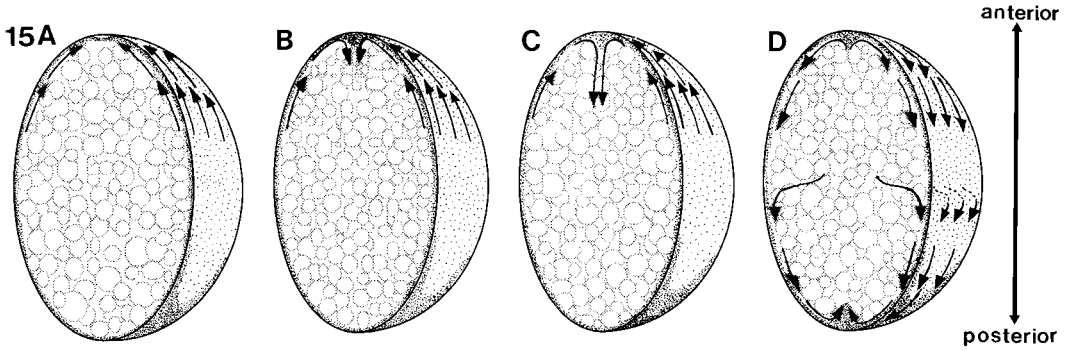
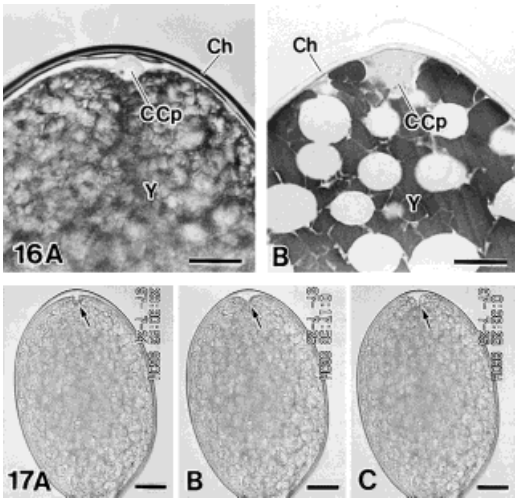


Fig. 15. *Ephemera japonica*. Diagrammatic representation of the yolk stream of the egg in association with the fertilization (**A–D**). Arrows represent the yolk stream. See text.

The cleavage nuclei migrate centrifugally and some nuclei reach the egg surface or periplasm by the fifth cleavage stage.

After the sixth cleavage, divisional synchrony diminishes and various phases of nuclei are encountered. In the sixth cleavage stage, many cleavage nuclei are present in the periplasm (Figs. 12, 14M). Thereafter, the peripheral cleavage nuclei undergo radial divisions. As a result of eight cleavages, about 250 cleavage nuclei arrive at the periplasm to form the syncytial blastoderm, i.e., the blastema (Fig. 14N). Cell membranes soon appear between the syncytial blastodermal nuclei and the blastoderm s. str. is completed (Figs. 13, 14O). About ten cleavage nuclei are left behind in the yolk and they are the primary yolk cells.

Thick posterior and thin anterior areas are already distinguishable in the newly formed blastoderm (Figs. 13, 14O). The former is the embryonic area, the cells of which concentrate at the posterior pole of the egg to form the germ disc. The latter is the extraembryonic area, the cells of which become more flattened to form the serosa (Fig. 14; Tojo and Machida, '97b).



Figs. 16, 17. *Ephemera japonica*. Activated unfertilized eggs, which were dissected out of subimagos, 2–3 hours after activation.

Fig. 16. Anterior region of eggs. CCp, condensed cytoplasm; Ch, chorion; Y, yolk. Bars = 10 µm. **A:** Living eggs. A depression of yolk is observed to be formed at the pole. **B:** Sectioned egg. Condensed cytoplasm devoid of male pronucleus can be seen at the anterior pole of the egg.

Fig. 17. Time-lapse VTR observation of an activated unfertilized egg (A–C). The egg was activated about 135 minutes before A (21:15, July 24, 1997). A depression of yolk appears at the anterior pole of eggs (**A**), deepens (**B**), and finally attains its maximum depth (**C**). Arrows, depression of yolk or condensed cytoplasm. Bars = 20 µm.

DISCUSSION

It is quite striking that in the early embryonic development of *Ephemera japonica* an extensive and circuitous migration of the male pronucleus is involved in the fertilization process. The male pronucleus approaches the female pronucleus not in a straight path, but circuitously. Its path is first anteriorwards in the periplasm from the entrance (i.e., micropyle) on the middorsal side of the egg to the anterior pole of the egg. It then changes its direction, posteriorwards in the yolk along the egg long axis to the site of syngamy, near the center of the egg.

Time-lapse VTR reveals that in the prefertilization egg of *Ephemera japonica*, a yolk

stream moves in accord with the migration of the male pronucleus in time and direction. Hence, we conclude that migration of the male pronucleus in *Ephemera japonica* is directed by or closely related to the observed yolk stream. The yolk stream is also generated in activated unfertilized eggs, a result implying that the yolk stream occurs regardless of the entry of sperm or the presence of a male pronucleus and that it may be intrinsic to the egg. However, the mechanism generating the yolk stream of *Ephemera japonica* is unknown. Investigations, especially of the cytoskeletal system, are needed to elucidate this issue. Some recent developmental biological works on *Drosophila* (e.g., Williams et al., '95, '97), especially using a mutation in the gene encoding the kinesin-like protein, clearly demonstrated that the cytoskeletal system plays the most important part for the proper behavior of male and female pronuclei at the fertilization.

Rempel and Church ('65) found a cytoplasmic stream in the early eggs of a meloid coleopteran *Lytta viridana* by examination of sectioned materials. They admitted the possibility that the yolk stream is closely related to the movement of the male pronucleus. The cytoplasmic stream of *Lytta viridana* strikingly resembles the yolk stream of *Ephemera japonica* in direction and phase. Our findings may thus verify Rempel and Church's correlation of male pronuclear movement and cytoplasmic streaming.

It may be interesting that a unique behavior of the male pronucleus in association with fertilization, which may be controlled by or is closely related to the extensive yolk or cytoplasmic stream, is commonly found in both a primitive mayfly *Ephemera japonica* and a remote advanced coleopteran *Lytta viridana*. The phylogenetic implication of this type of male pronuclear behavior, however, remains to be clarified. This behavior has not been reported in any other insect groups.

Sauer ('66) used time-lapse cinematography to observe the fertilization of a cricket *Gryllus domesticus*. He found that in this species, the approach of male and female pronuclei, which does not involve an extensive circuitous migration of the male pronucleus as observed in *Ephemera japonica* but is fulfilled in the shortest way, is caused by contraction waves of the yolk.

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LITERATURE CITED

Ando, H., and T. Kawana (1956) Embryology of mayfly (*Ephemera strigata* Eaton) as studied by external observation. Kontyû *24*:224–232 [in Japanese with English summary].

Bohle, H.W. (1969) Untersuchungen über die Embryonale Diapause bei *Baëtis vernus* und *Baëtis rhodani*. Zool. Jb. Anat. *86*:493–575.

Boudreaux, H.B. (1979) Arthropod Phylogeny With Special Reference to Insects. New York: John Wiley and Sons, pp. 320.

Hennig, W. (1969) Die Stammesgeschichte der Insekten. Frankfurt am Main: Kramer.

Heymons, R. (1896a) Grundzüge der Entwicklung und des Körperbaues von Odonaten und Ephemeriden. Berlin: Anhang zu den Abhandlungen der Köiglichen Akademie der Wissenschaften zu Berlin.

Heymons, R. (1896b) Mitteilungen über die Lebensweise und Entwicklung von *Ephemera vulagata* L. Sb. Ges. Naturf. Freunde, Berlin *1896*:81–96.

Heymons, R. (1896c) Zur Morphologie des Abdominalanhänge bei den Insekten. Morphol. Jb. *24*:178–204.

Joly, M.N. (1876) On the embryogeny of the Ephemerae, especially that of *Palingenia virgo*, Oliv. Ann. Mag. Nat. Hist. Lond., Ser. 4. *17*:481–484.

Kristensen, N.P. (1975) The phylogeny of the hexapod 'orders'. A critical review of recent accounts. Z. Zool. Syst. Evolut.-forsch. *13*:1–44.

Machida, R., T. Nagashima, and H. Ando (1994a) Embryonic development of the jumping bristletail *Pedetontus unimaculatus* Machida, with special reference to embryonic membranes (Hexapoda: Microcoryphia, Machilidae). J. Morphol. *220*:147–165.

Machida, R., T. Nagashima, and T. Yokoyama (1994b) Mesoderm segregation of a jumping bristletail, *Pedetontus unimaculatus* Machida (Hexapoda, Microcoryphia), with a note on an automatic vacuum infiltrator. Proc. Arthropod. Embryol. Soc. Jpn. *29*:23–24 [in Japanese].

Murphy, H.E. (1922) Notes on the biology of some of North American species of mayflies. Bull. Lloyd. Libr. Entomol. *2*:3–39.

Rempel, J.G., and N.S. Church (1965) The embryology of *Lytta viridana* Le Conte (Coleoptera: Meloidae) I. Maturation, fertilization, and cleavage. Can. J. Zool. *43*:915–925.

Sauer, H. W. (1966) Zeitraffer-mikro-film-analyse embryonaler Differenzierungsphasen von *Gryllus domesticus*. Z. Morphol. Ökol. Tiere *56*:143–251.

Tojo, K., and R. Machida (1996) Formation of proctodaeum and terminal abdominal structures of *Ephemera japonica* McLachlan (Insecta: Ephemeroptera). Proc. Arthropod. Embryol. Soc. Jpn. *31*:29–32 [in Japanese].

Tojo, K., and R. Machida (1997a) Embryogenesis of *Ephemera japonica* McLachlan (Insecta: Ephemeroptera). Proc. Arthropod. Embryol. Soc. Jpn. *32*:25–28.

Tojo, K., and R. Machida (1997b) Embryogenesis of the mayfly *Ephemera japonica* McLachlan (Insecta: Ephemeroptera, Ephemeridae), with special reference to abdominal formation. J. Morphol. *234*:97–107.

Tojo, K., and R. Machida (1998) Early embryonic development of *Ephemera japonica* McLachlan (Insecta: Ephemeroptera). Proc. Arthropod. Embryol. Soc. Jpn. *33*:31–33 [in Japanese].

Tojo, K., and R. Machida (1999) Egg structures of Japanese ephemerid species (Ephemeroptera). Entomol. Sci. (in press).

Tsui, P.T.P., and W.L. Peters (1974) Embryonic development, early instar morphology, and behavior of *Tortopus inceratus* (Ephemeroptera: Polymitarcidae). Florida Entomol. *54*:349–356.

Williams, B.C., M.F. Riedy, E.V. Williams, M. Gatti, and M.L. Goldberg (1995) The *Drosophila* kinesin-like protein KLP3A is a midbody component required for central spindle assembly and initiation of cytokinesis. J. Cell Biol. *129*:709–723.

Williams, B.C., A.F. Dernburg, J. Puro, S. Nokkala, and M. L. Goldberg (1997) The *Drosophila* kinesin-like protein KLP3A is required for proper behavior of male and female pronuclei at fertilization. Development *124*: 2365–2376.

Wolf, B.E. (1960) Zur Karyologie der Eireifung und Furchung bei *Cloeon dipterum* L. (Ephemerida: Baetidae). Biol. Zbl. *79*:153–198.